PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/55, 1/15, 1/19, 9/18, C11D 3/386, C12N 15/62 — A1

(11) International Publication Number: WO 94/14963

(43) International Publication Date: 7 July 1994 (07.07.94)

(21) International Application Number: PCT/EP93/03550

(22) International Filing Date: 9 December 1993 (09.12.93)

(30) Priority Data:
92204025.8 18 December 1992 (18.12.92) NL

(71) Applicant *(for AU BB CA GB IE LK MN MW NZ SD only)*: UNILEVER PLC [GB/GB]; Unilever House, Blackfriars, London EC4 4BQ (GB).

(71) Applicant *(for all designated States except AU BB CA GB IE LK MN MW NZ SD)*: UNILEVER N.V. [NL/NL]; Weena 455, NL-3013 AL Rotterdam (NL).

(72) Inventors: EGMOND, Maarten, Robert; Catharijnesingel 114, NL-3511 GW Utrecht (NL). VAN DER HIJDEN, Hendrikus, Theodorus, W., M.; Aida 64, NL-2907 KB Capelle a/d Ijssel (NL). MUSTERS, Wouter; Wipperspark 138, NL-3141 RD Maassluis (NL). PETERS, Hans; Saffierstraat 12, NL-3051 XV Rotterdam (NL). VERRIPS, Cornelis, Theodorus; Hagedoorn 18, NL-3142 KB Maassluis (NL). DE VLIEG, Jakob; Kastanjedal 32, NL-3142 AP Maassluis (NL).

(74) Common Representative: UNILEVER N.V. PATENT DIVISION; Patent Division, P.O. Box 137, NL-3130 AC Vlaardingen (NL).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MODIFIED CUTINASES, DNA, VECTOR AND HOST

(57) Abstract

Eukaryotic Cutinase variants having improved lipolytic activity are provided, wherein the amino acid sequence has been modified in such a way that the hydrophobicity at the surface of the enzyme has been increased. In particular, variants of *Fusarium solani pisi* Cutinase are provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

MODIFIED CUTINASES, DNA, VECTOR AND HOST

TECHNICAL FIELD

The present invention generally relates to the field of lipolytic enzymes. More in particular, the invention is concerned with lipolytic enzymes which have been modified by means of recombinant DNA techniques, with methods for their production and with their use, particularly in enzymatic detergent compositions.

BACKGROUND AND PRIOR ART

Lipolytic enzymes are enzymes which are capable of hydrolysing triglycerides into free fatty acids and diglycerides, monoglycerides and eventually glycerol. They can also split more complex esters such as cutin layers in plants or sebum of the skin. Lipolytic enzymes are used in industry for various enzymatic processes such as the inter- and trans-esterification of triglycerides and the synthesis of esters. They are also used in detergent compositions with the aim to improve the fat-removing properties of the detergent product.

The most widely used lipolytic enzymes are lipases (EC 3.1.1.3). For example, EP-A-258 068 and EP-A-305 216 (both Novo Nordisk) both describe production of fungal lipases via heterologous host micro-organisms by means of rDNA techniques, especially the lipase from Thermomyces lanuginosus/Humicola lanuginosa. EP-A-331 376 (Amano) describes lipases and their production by rDNA techniques, and their use, including an amino acid sequence of lipase from Pseudomonas cepacia. Further examples of lipases produced by rDNA technique are given in WO-A-89/09263 and EP-A-218 272 (both Gist-Brocades). In spite of the large number of publications on lipases and their modifications, only the lipase from Humicola lanuginosa has so far found wide-spread commercial application as additive for detergent products under the trade name Lipolase (TM).

A characteristic feature of lipases is that they exhibit interfacial activation. This means that the enzyme activity is much higher on a substrate which has formed

interfaces or micelles, than on fully dissolved substrate. Interface activation is reflected in a sudden increase in lipolytic activity when the substrate concentration is raised above the critical micel concentration (CMC) of the substrate, and interfaces are formed. Experimentally this phenomenon can be observed as a discontinuity in the graph of enzyme activity versus substrate concentration.

The mechanism of interfacial activation in lipases has been interpreted in terms of a conformation change in the protein structure of the lipase molecule. In the free, unbound state, a helical lid covers the catalytic binding site. Upon binding to the lipid substrate, the lid is displaced and the catalytic site is exposed. The helical lid is also believed to interact with the lipid interface, thus allowing the enzyme to remain bound to the interface.

WO-A-92/05249 (Novo Nordisk) discloses genetically modified lipases, in particular the lipase from Humicola lanuginosa, which have been modified at the lipid contact zone. The lipid contact zone is defined in the application as the surface which in the active form is covered by the helical lid. The modifications involve deletion or substitution of one or more amino acid residues in the lipid contact zone, so as to increase the electrostatic charge and/or decrease the hydrophobicity of the lipid contact zone, or so as to change the surface conformation of the lipid contact zone. This is achieved by deleting one or more negatively charged amino acid residues in the lipid contact zone, or substituting these residues by neutral or more positively charged amino acids, and/or by substituting one or more neutral amino acid residues in the lipid contact zone by positively charged amino acids, and/or deleting one or more hydrophillic amino acid residues in the lipid contact zone, or substituting these residues by hydrophobic amino acids.

Cutinases are a sub-class of enzymes (EC 3.1.1.50), the wax ester hydrolases. These enzymes are capable of degrading cutin, a network of esterified long-chain fatty acids and fatty alcohols which occurs in plants as a protective coating on leaves and stems. In addition, they

possess some lipolytic activity, i.e. they are capable of hydrolysing triglycerides. Thus they can be regarded as a special kind of lipases. Contrary to lipases, however, cutinases do not exhibit any substantial interfacial activation.

Cutinases can be obtained from a number of sources, such as plants (e.g. pollen), bacteria and fungi. Because of their fat degrading properties, cutinases have been proposed as ingredients for enzymatic detergent compositions. For example, WO-A-88/09367 (Genencor) suggests combinations of a surfactant and a substantially pure bacterial cutinase enzyme to formulate effective cleaning compositions. Disclosed are detergent compositions comprising a cutinase obtained from the Gram negative bacterium Pseudomonas putida ATCC 53552. However, in the more recent European patent application EP-A-476 915 (Clorox), it is disclosed that the same enzyme - which is then referred to as a lipase - is no more effective than other lipases in removing oil stains from fabrics, when used by conventional methods.

Recently, the three-dimensional structure has been determined of a cutinase from Fusarium solani pisi (Martinez et al. (1992) Nature 356, 615-618). It was found that this cutinase does not possess a helical lid to cover the catalytic binding site. Instead, the active site serine residue appears to be accessible to the solvent. These findings appear to confirm the present theory about the mechanism of interfacial activation in lipases.

The cutinase gene from Fusarium solani pisi has been cloned and sequenced (Ettinger et al., (1987) Biochemistry 26, 7883-7892). WO-A-90/09446 (Plant Genetics Systems) describes the cloning and production of this gene in E. coli. The cutinase can efficiently catalyse the hydrolysis and the synthesis of esters in aqueous and non-aqueous media, both in the absence and the presence of and interface between the cutinase and the substrate. On the basis of its general stability, it is suggested that this cutinase could be used to produce cleaning agents such as laundry detergents and other specialized fat dissolving preparations such as

cosmetic compositions and shampoos. A way to produce the enzyme in an economic feasible way is not disclosed, neither are specific enzymatic detergent compositions containing the cutinase.

Because of this characteristic feature, i.e. the absence of interfacial activation, we define for the purpose of this patent application Cutinases as lipolytic enzymes which exhibit substantially no interfacial activation. Cutinases therefor differ from classical lipases in that they do not possess a helical lid covering the catalytic binding site.

As mentioned above, only the lipase derived from Humicola lanuginosa has so far found wide-spread commercial application as additive for detergent products under the trade name Lipolase (TM). In his article in Chemistry and Industry 1990, pages 183-186, Henrik Malmos notes that it is known that generally the activity of lipases during the washing process is low, and Lipolase (TM) is no exception. During the drying process, when the water content of the fabric is reduced, the enzyme regains its activity and the fatty stains are hydrolysed. During the following wash cycle the hydrolysed material is removed. This also explains why the effect of lipases is low after the first washing cycle, but significant in the following cycles. Thus, there is still a need for lipolytic enzymes which exhibit any significant activity during the washing process.

We have found that Cutinases, in particular the cutinase from Fusarium solani pisi, exhibit a clear in-the-wash effect. However, there is still a need for Cutinases having improved in-the-wash lipolytic activity and for methods for producing such enzymes.

The purpose of the present invention is to provide Cutinases, which have been modified so as to improve their performance, especially their in-the-wash lipolytic activity.

We have now surprisingly found that the lipolytic activity of eukaryotic Cutinase enzymes, more in particular of Cutinases from Fusarium solani pisi, Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe

grisea, may be improved by modifying the amino acid sequence in such way that the hydrophobicity at the surface of the enzyme has been increased.

DEFINITION OF THE INVENTION

A Cutinase variant of a parent Cutinase, wherein the amino acid sequence has been modified in such way that the hydrophobicity at the surface of the enzyme has been increased. Preferably, the hydrophobicity at the surface of the enzyme has been increased so as to form an enlarged lipid contact zone.

DESCRIPTION OF THE INVENTION

The invention relates to variants of Cutinase enzymes. As discussed above, Cutinases can be obtained from a number of sources, such as plants (e.g. pollen), bacteria and fungi. The Cutinase to be used as parent Cutinase or starting material in the present invention for the modification by means of recombinant DNA techniques, is chosen from the group of eukaryotic Cutinases. Eukaryotic Cutinases can be obtained from various sources, such as plants (e.g. pollen), or fungi.

The group of (eukaryotic) fungal Cutinases appears to comprise two families with different specificities, leaf-specificity and stem-specificity. Cutinases with leaf-specificity tend to have an acidic or neutral pH-optimum, whereas Cutinases with stem-specificity tend to have an alkaline pH-optimum. Cutinases having an alkaline pH-optimum are more suitable for use in alkaline built detergent compositions such as heavy duty fabric washing powders and liquids. Cutinase having an acidic to neutral pH-optimum are more suitable for light duty products or rinse conditioners, but also for industrial cleaning products.

In the following Table I, four different Cutinases with stem-specificity are listed, together with their pH-optima.

TABLE I

Examples of cutinases with stem-specificity	pH-optimum
Fusarium solani pisi	9
Fusarium roseum culmorum	10
Rhizoctonia solani	8.5
Alternaria brassicicola (PNBase I)	9

Especially preferred in the present invention are Cutinases which can be derived from wild type Fusarium solani pisi (Ettinger et al. 1987). When used in certain detergent compositions, this Cutinase exhibits clear "in-the-wash" effects.

Also suitable as parent Cutinase or starting material in the present invention for the modification by means of recombinant DNA techniques, are Cutinases having a high degree of homology of their amino acid sequence to the Cutinase from Fusarium solani pisi. Examples are the Cutinases from Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea. In Figure 12 the partial amino acid sequences of these Cutinases are shown and it can be seen that there is a high dergee of homology.

Alternative to the improvement of Fusarium solani pisi cutinase by modification of its gene, genetic information encoding Cutinases from other eukaryotic organisms can be isolated using 5'- and 3'- DNA probes derived from Fusarium solani pisi, Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea cDNA encoding (pro)cutinase and probes recognizing conserved sequences in other lipolytic enzymes and if necessary, using these probes to multiply cDNA's derived from messenger RNA's (mRNA's) of Cutinase producing eukaryotic cells using the Polymerase Chain Reaction or PCR technology (see, for example WO-A-92/05249). After cloning and expression the thus obtained Cutinases encoding genes in E. coli according standard procedures, the Cutinases are tested on their performance in (fatty) soil removal under appropriate conditions. In this way a number of natural occurring variants of the above mentioned Cutinases can be obtained

with improved in-the-wash performance. Moreover, the sequences of these natural occurring Cutinases provide an excellent basis for further protein engineering of Fusarium solani pisi cutinase.

On the basis of new ideas about the factors determining the activity of "in-the-wash" lipolytic enzymes and careful inspection of the 3D structure of Fusarium solani pisi cutinase we have found a number of possibilities how to improve the performance of this cutinase and Cutinases in general by means of recombinant DNA techniques.

As Cutinases differ fundamentally from lipases like Lipolase (TM), the interaction between Cutinase the substrate (the lipid interface) will be based on principles different from lid opening and exposure of a hydrophobic area that can bind the substrate (Brzozowski et al. (1991) Nature 351, 491-494).

The present invention shows that Cutinases can be modified in such a way that the interaction with the substrate can be improved without forming such large hydrophobic areas on the surface of the modified Cutinase that the Cutinase molecules start to aggregate. The enlargement of the hydrophobic surface can be obtained by introducing hydrophobic amino acids like alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and tyrosine, and methionine and to a lesser degree glutamic acid, glutamine and histidine, provided that the hydrophobic side chains of these amino acids are not buried in the hydrophobic core of the Cutinase. Methionine is not normally considered to be a hydrophobic amino acid. However, when integrated at certain positions, methionine can effectively contribute to the increased hydrophobicity at the surface of the Cutinase molecule.

In some cases it was found to be beneficial to introduce beside the hydrophobic amino acids also charged amino acids to avoid aggregation of the enzyme. Surprisingly, we have found that when integrated at certain positions in the Cutinase molecule, positively charged amino acids like lysine and arginine can also give enlargement of the

hydrophobic surface area. This is limited to those positions in the Cutinase molecule where the methylene groups present in lysine and arginine can not be buried in the molecule. The advantage of using lysine or arginine is that the amino- or imido groups increase the probability that the methylene groups will be exposed and therefor will be able to interact with the lipid phase.

Lysine and Arginine are not normally considered to be hydrophobic amino acids. However, the atoms forming the side chains of these residues contain a large number of hydrophobic atoms (in the methylene moieties) which may interact with the lipid phase. In fact, the size of the hydrophobic part of a lysine residue is comparable to that of a valine residue.

If other intrinsic properties of the Cutinase are negatively affected by the introduction of the positive charge, this may be compensated by the introduction of a compensating negative charge or deletion of a positive charge at the surface in or near that part of the Cutinase molecule which interacts with the lipid phase.

Inspection of the hydrophobicity of the surface of Fusarium solani pisi cutinase around the active site shows that the hydrophobicity is not optimal. To improve this certain amino acids in this area residues should be replaced by more bulky hydrophobic residues.

In order to get a better understanding of the relationship between structure and function in lipolytic enzymes, we have carefully studied the three-dimensional (3D) structures of a number of such enzymes. When these structures had not been published, we derived the structures by means of molecular modelling techniques.

The 3D structure of the Rhizomucor miehei lipase has been determined by X-ray crystallographic methods (Brady et al. (1990) Nature 343, 767-770, Brzozowski et al. (1991) Nature 351, 491-494, Derewenda et al. (1992) Biochemistry 31, 1532-1541). The active-site Ser 144, belonging to a Ser-His-Asp protease-like catalytic triad, is buried under a short helical lid (residues 85-91). The structure in which the

active-site Ser is buried is referred to below as the "closed" conformation of the enzyme. It is believed that the adsorption at the oil-water interface is associated by a movement of the helical lid. As a consequence of this movement the active-site Serine becomes exposed and the hydrophobic area around the active-site increases. It is believed that the "open" conformation corresponds to the activated enzyme adsorbed at the oil-water interface.

The Cα-coordinates of the "closed" form of the fungus Rhizomucor miehei lipase have been deposited in the Protein Data Bank at Brookhaven. Elaborate computational methods were used for generating full protein coordinates (backbone and side-chains) of the Rhizomucor miehei lipase. A crude starting model of the Rhizomucor miehei lipase was generated by applying the computational procedures described in S. Wodak et al. (1989) Protein Engineering 2, 335-345. This method is implemented in the SYBYL molecular modelling software package (TRIPOS associates, Inc. St. Louis, Missouri). Subsequently, the model was refined by applying energy minimization (EM) and molecular dynamics (MD) techniques as implemented in the BIOSYM molecular modelling software package (BIOSYM, San Diego, California). During EM and MD refinement of the model a knowledge-based approach was applied. The model was simultaneously optimized for the detailed energy terms of the potential energy function and known structural criteria. Model quality was assessed by criteria such as number and quality of hydrogen bonds, hydrogen bonding patterns in the secondary structure elements, the orientation of peptide units, the values of and main chain dihedral angles, the angle of interaction of aromatic groups and the sizes of cavities. Moreover, the model was checked for inappropriately buried charges, extremely exposed hydrophobic residues and energetically unfavourable positions of disulphide bridges. Relevant side-chain rotamers were selected from the Ponder & Richards rotamer library (Ponder et al. (1987) J.Mol.Biol. 193, 775-791). The final choice of a particular side-chain rotamer from this library was based on structural criteria

evaluations as mentioned above. MD was used to anneal the side-chain atoms into position. Elaborate examination of the model structure for consistency with known structural properties and EM and MD calculations to optimize structural characteristics allow to generate a reliable full atom model of the Rhizomucor miehei lipase. The "open" conformation of Rhizomucor miehei lipase was obtained by applying an MD computer simulation in which a C_{10}-triglyceride was docked into the active site of the lipase. Elaborate comparison to the published conformational characteristics of the open structure (Derewenda et al. Biochemistry (1992) 31, 1532-1541) showed that the computer model of the "open" conformation which was obtained in this way, is essentially the same.

Starting from the known 3D structure of the fungus Rhizomucor miehei lipase, the "open" and "closed" 3D-structures of Humicola langinosa lipase were obtained by applying rule-based comparative modelling techniques as implemented in the COMPOSER module of the SYBYL molecular modelling software package (TRIPOS associates, Inc. St. Louis, Missouri). The obtained model of Humicola langinosa lipase was refined by the same computational procedures as mentioned above.

The part of the lipase molecule which is involved in the adsorption of the substrate onto the enzyme was identified by comparing the three-dimensional (3D) structures of the fungus Rhizomucor miehei lipase and the Humicola langinosa lipase.

Starting from the known 3D structure of the Fusarium solani pisi cutinase, the 3D-structure of the cutinase from Colletotrichum gloeosporiodes was obtained by applying rule-based comparative modelling techniques as implemented in the COMPOSER module of the SYBYL molecular modelling software package (TRIPOS associates, Inc. St. Louis, Missouri). The obtained model of the Colletotrichum gloeosporiodes cutinase was refined by the same computational procedures as mentioned above.

From the three-dimensional structures of the lipolytic enzymes listed below in Table II, it is was unexpectedly observed that one can define a particular vector which is the least-square fit through the Cα-atoms of residues 116 to 120 of the Fusarium solani pisi cutinase. This vector is essentially perpendicular to the surface where the interaction with the substrate occurs.

From the following Table II it follows that when the primary sequences of a number of lipolytic enzymes from different sources are compared, the amino acid residues 116 to 120 of the Cutinase ex Fusarium solani pisi appear to be located in an area having a large extent of functional homology. The alignment can be guided by the use of the consensus sequence Gly-(His/Tyr)-Ser-X-Gly for lipolytic enzymes.

TABLE II

Humicola lanuginosa lipase	YRVVFTGHSLGGALATVAGADLRGNGY
Mucor miehei lipase	YKVAVTGHSLGGATALLCALGLYQREE
human pancreas lipase	SNVHVIGHSLGAHAAGEAGRRTNGTIG
Fusarium s.p. cutinase	ATLIAGGYSQGAALAAASIEDLDSAIR
C. gloeosporiodes cutinase	AAIVSGGYSQGTAVMAGSISGLSTTIK

Therefor we have used the vector through the amino acid residues 116 to 120 in the Fusarium solani pisi cutinase molecule to define the part of the Cutinase molecule in which the amino acid modifications should be made in order to obtain a Cutinase having improved in-the-wash activity. The following Table III gives the structure of the neighbouring amino acids for the lipolytic enzymes shown in Table II.

TABLE III

	strand	strand	helix	act.site
H. lanuginosa lip.	138-141	142 Ser*146	147-159	his258
Mucor miehei lipase	136-139	(fit:140-Ser*144)	145-157	his257
human pancreas lip.	144-147	(fit:148-Ser*152)	153-165	his263
F.s.pisi cutinase	112-115	(fit:116-Ser*120)	121-133	his188
C. gloe. cutinase	112-115	(fit:116-Ser*120)	121-133	his188

Ser* = active site Serine

The invention in one of its aspects provides a modified Cutinase enzyme having improved in-the-wash lipolytic activity wherein the amino acid sequence has been modified in such way that the hydrophobicity at the surface of the enzyme has been increased. Preferably, the hydrophobicity at the surface of the enzyme adjacent to the lipid contact zone has been increased so as to form an enlarged lipid contact zone.

The increase in surface hydrophobicity of the Cutinase can be achieved by replacing one or more amino acid residues by amino acid residues selected from the group consisting of alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and tyrosine, methionine, glutamic acid, glutamine and histidine. Preferred are valine, leucine, isoleucine, phenylalanine, tryptophan and methionine.

It was found to be advantageous to modify the amino acid sequence in such way that in addition to the increase in hydrophobicity at the surface, one or more positive charges have been introduced by introduction of one or more lysine or arginine residues.

Preferably, the modified residues are located in that part of the molecule which is defined by the vector which is the least-square fit through the Cα-atoms of residues 116 to 120 of the Fusarium solani pisi cutinase, or the corresponding Cα-atoms of a different Cutinase, and the plane perpendicular to said vector and containing the Cα-atom of residue 117, or the corresponding Cα-atom of a different Cutinase.

As said before, for the three-dimensional structure of the Cutinase from Fusarium solani pisi has been published. In that case it will be clear which modification will lead to modifications within the scope of this invention. In case the thee-dimensional structure of a particular Cutinase is not yet known, it will nevertheless be possible by alignment of the amino acid sequence with a known sequence (see Fig.12), guided by the consensus sequence Gly-(His/Tyr)-Ser-X-Gly for lipolytic enzymes, to arrive at suitable modifications within

the scope of the present invention. Preferably, molecular modelling techniques are also used in this process.

The Cutinases variants produced according to the invention can bring advantage in enzyme activity, when used as part of detergent or cleaning compositions. In particular, they were found to possess an improved in-the-wash performance during the main cycle of a wash process. By in-the-wash performance during the main cycle of a wash process, it is meant that a detergent composition containing the enzyme is capable of removing a significant amount of oily soil from a soiled fabric in a single wash process in a European type of automatic washing machine, using normal washing conditions as far as concentration, water hardness, temperature, are concerned. It should be born in mind that under the same conditions, the conventional commercially available lipolytic enzyme Lipolase (TM) ex Novo Nordisk does not appear to have any significant in-the-wash effect on oily soil.

The in-the-wash effect of an enzyme on oily soil can be assessed using the following assay. New polyester test having a cotton content of less than 10% are prewashed using an enzyme-free detergent product such as the one given below, and are subsequently thoroughly rinsed. Such unsoiled cloths are then soiled with olive oil or another suitable, hydrolysable oily stain. Each tests cloth (weighing approximately 1 g) is incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. The wash liquor contains the detergent product given below at a dosage of 1 g per litre. The bottles are agitated for 30 minutes in a Miele TMT washing machine filled with water and using a normal 30°C main wash programme. The Cutinase variant is preadded to the wash liquor at 3 LU/ml. The control does not contain any enzyme. The washing powder has the following composition (in % by weight):

Ethoxylated alcohol nonionic surfactant	9.5
Sodium sulphate	38.6
Sodium carbonate	40.4
Sodium silicate ($Na_2O:Si_2O$ = 2.4)	7.3
Water	4.2

As nonionic surfactant we used C_{12}-C_{15} ethoxylated alcohol 10.5-13 EO, but the nature of the ethoxylated alcohol nonionic can vary within wide limits.

After washing, the cloths are thoroughly rinsed with cold water and dried in a tumble dryer with cold air, and the amount of residual fat is assessed. This can be done in several ways. The common method is to extract the test cloth with petroleum ether in a Soxhlet extraction apparatus, distilling off the solvent and determining the percentage residual fatty material as a fraction of the initial amount of fat on the cloth by weighing.

According to a second, more sensitive method, brominated olive oil is used to soil the test cloths (Richards, S., Morris, M.A. and Arklay, T.H. (1968), Textile Research Journal 38, 105-107). Each test cloth is then incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. A series of bottles is then agitated in a washing machine filled with water and using a normal 30°C main wash programme. After the main wash, the test cloths are carefully rinsed in cold water during 5 seconds. Immediately after the rinse, the test cloths dried in a dryer with cold air. After drying the amount of residual fat can be determined by measuring the bromine content of the cloth by means of X-ray fluorescence spectrometry. The fat removal can be determined as a percentage of the amount which was initially present on the test cloth, as follows:

$$\% \text{ Soil removal} = \frac{\text{Bromine}_{bw} - \text{Bromine}_{aw}}{\text{Bromine}_{bw}} * 100\ \%$$

wherein: Bromine_{bw} denotes the percentage bromine on the cloth before the wash and Bromine_{aw} the percentage bromine after the wash.

A further method of assessing the enzymatic activity is by measuring the reflectance at 460 nm according to standard techniques.

In the context of this invention, a modified, mutated or mutant enzyme or a variant of an enzyme means an enzyme that has been produced by a mutant organism which is expressing a mutant gene. A mutant gene (other than one

containing only silent mutations) means a gene encoding an enzyme having an amino acid sequence which has been derived directly or indirectly, and which in one or more locations is different, from the sequence of a corresponding parent enzyme. The parent enzyme means the gene product of the corresponding unaltered gene. A silent mutation in a gene means a change or difference produced in the polynucleotide sequence of the gene which (owing to the redundancy in the codon-amino acid relationships) leads to no change in the amino acid sequence of the enzyme encoded by that gene.

A mutant or mutated micro-organism means a micro-organism that is, or is descended from, a parent micro-organism subjected to mutation in respect of its gene for the enzyme. Such mutation of the organism may be carried out either (a) by mutation of a corresponding gene (parent gene) already present in the parent micro-organism, or (b) by the transfer (introduction) of a corresponding gene obtained directly or indirectly from another source, and then introduced (including the mutation of the gene) into the micro-organism which is to become the mutant micro-organism. A host micro-organism is a micro-organism of which a mutant gene, or a transferred gene of other origin, forms part. In general it may be of the same or different strain or species origin or descent as the parent micro-organism.

In particular, the invention provides mutant forms of the Fusarium solani pisi cutinase disclosed in WO-A-90/09446 (Plant Genetics Systems), and of the Cutinases from Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea. These Cutinase variants can be produced by a rDNA modified micro-organism containing a gene obtained or made by means of rDNA techniques.

Once the amino acid residues have been identified which are located in that part of the molecule which is defined by the vector which is the least-square fit through the Cα-atoms of residues 116 to 120 of the Fusarium solani pisi cutinase, or the corresponding Cα-atoms of a different Cutinase, and the plane perpendicular to said vector and containing the Cα-atom of residue 117, or the corresponding

Cα-atom of a different Cutinase, one can attempt to modify the amino acid sequence by introduction of suitable amino acids at one or more of the identified positions, for example mutation N712K relative to the sequence of Fusarium solani pisi cutinase or a homologue thereof.

It will be clear to the skilled man that such modifications will affect the structure of the Cutinase. Obviously, modifications are preferred which do not affect the electrostaic charge around the active site too much.The inventors have developed the necessary level of understanding of the balance between the inevitable distortion of the conformation of the enzyme and the benefit in increased enzyme activity, which makes is possible to predict and produce successful Cutinase variants with a high rate of success.

In the following Table IV and elsewhere in this specification, amino-acids and amino acid residues in peptide sequences are indicated by one-letter and three-letter abbreviations as follows:

TABLE IV

A	= Ala	= Alanine		V	= Val	= Valine	
L	= Leu	= Leucine		I	= Ile	= Isoleucine	
P	= Pro	= Proline		F	= Phe	= Phenylalanine	
W	= Trp	= Tryptophan		M	= Met	= Methionine	
G	= Gly	= Glycine		S	= Ser	= Serine	
T	= Thr	= Threonine		C	= Cys	= Cysteine	
Y	= Tyr	= Tyrosine		N	= Asn	= Asparagine	
Q	= Gln	= Glutamine		D	= Asp	= Aspartic Acid	
E	= Glu	= Glutamic Acid		K	= Lys	= Lysine	
R	= Arg	= Arginine		H	= His	= Histidine	

In this specification, a mutation present in the amino acid sequence of a protein, and hence the mutant protein itself, may be described by the position and nature of the mutation in the following abbreviated way: by the identity of an original amino acid residue affected by the mutation; the site (by sequence number) of the mutation; and by the identity of the amino acid residue substituted there

in place of the original. If there is an insertion of an extra amino acid into the sequence, its position is indicated by one or more subscript letters attached to the number of the last preceding member of the regular sequence or reference sequence.

For example, a mutant characterised by substitution of Asparagine by Lysine in position 172 is designated as: Asn172Lys or N172K. A (hypothetical) insertion of an additional amino acid residue such as proline after the Asparagine would be indicated as Asn172AsnPro or N172NP, alternatively as *172aP, with the inserted residue designated as position number 172a. A (hypothetical) deletion of Asparagine in the same position would be indicated by Asn172* or N172*. The asterisk stands either for a deletion or for a missing amino acid residue in the position designated, whether it is reckoned as missing by actual deletion or merely by comparison or homology with another or a reference sequence having a residue in that position.

Multiple mutations are separated by plus signs, e.g. N172K+S54I+A128F designates a mutant protein carrying three mutations by substitution, as indicated for each of the three mentioned positions in the amino acid sequence. The mutations given in the following table may be combined if desired.

The Table V given below shows certain useful examples of Cutinase variants according to the invention, based on the sequence of Cutinase from Fusarium solani pisi.

TABLE V

Variants of Fusarium solani pisi Cutinase.

T19V, G41A, T45K, T45P, *I49a, S54I, N58R, G75R, A76P, G82A, D83S, A85F, A85V, S92R, A93V, L99K, G100R, A127L, A128F, N172K, T173I, T179F, I183F, V184I, A185L, L189F, A190L, D194R, G197V, E201K

Preferred variants according to the invention are A85F, N172K and E201K of Fusarium solani pisi cutinase, and the corresponding variants of other Cutinases. An example of such a corresponding Cutinase variant is the variant

Asp172Lys or D172K derived from cutinase ex Colletotrichum gloeosporiodes.

According to a further aspect of the invention, there is provided a process for producing the Cutinase variants of the invention. Naturally occurring Cutinase producing micro-organisms are usually plant pathogens and these micro-organisms are not very suitable to act as host cell for modified Cutinases genes. Consequently, the genes coding for modified (pro)Cutinases were integrated in rDNA vectors that can be transferred into the preferred host micro-organism for rDNA technology. For this purpose rDNA vectors essentially similar to the rDNA vector described in WO-A-90/09446 can be used.

Naturally occurring Cutinase producing micro-organisms are not very suitable for fermentation processes. To improve the yield of the fermentation process a gene coding for improved Cutinases should be transferred into micro-organisms that can growth fast on cheap medium and are capable to synthesize and secrete large amounts of Cutinase. Such suitable rDNA modified (host micro-organisms) according to the present invention are bacteria, among others, Bacilli, Corynebacteria, Staphylococci and Streptomyces, or lower eukaryotes such as Saccharomyces cerevisiae and related species, Kluyveromyces marxianus and related species, Hansenula polymorpha and related species, and species of the genus Aspergillus. Preferred host micro-organisms are the lower eukaryotes, because these microorganisms are producing and secreting enzymes very well in fermentation processes and are able to glycolysate the Cutinase molecule. Glycosylation can contribute to the stability of the Cutinase in detergent systems.

The invention also provides genetic material derived from the introduction of modified eukaryotic Cutinase genes, e.g. the gene from Fusarium solani pisi, into cloning rDNA vectors, and the use of these to transform new host cells and to express the genes of the Cutinase variants in the new host cells.

Also provided by the invention are polynucleotides made or modified by rDNA technique, which encode such Cutinase variants, rDNA vectors containing such polynucleotides, and rDNA modified microorganisms containing such polynucleotides and/or such rDNA vectors. The invention also provides corresponding polynucleotides encoding the modified eukaryotic Cutinases, e.g. a polynucleotide having a base sequence that encodes a mature Cutinase variant, in which polynucleotide the final translated codon is followed by a stop codon and optionally having nucleotide sequences coding for the prepro- or pro-sequence of this Cutinase variant directly upstream of the nucleotide sequences coding for the mature Cutinase variant.

In such a polynucleotide, the Cutinase-encoding nucleotide sequence derived from the organism of origin can be modified in such a way that at least one codon, and preferably as many codons as possible, are made the subject of 'silent' mutations to form codons encoding equivalent amino acid residues and being codons preferred by a new host, thereby to provide in use within the cells of such host a messenger-RNA for the introduced gene of improved stability.

Upstream of the nucleotide sequences coding for the pro-or mature Cutinases, there can be located a nucleotide sequence that codes for a signal or secretion sequence suitable for the chosen host. Thus an embodiment of the invention relates to a rDNA vector into which a nucleotide sequence coding for a Cutinase variant or a precursor thereof has been inserted.

The nucleotide sequence can be derived for example from:

(a) a naturally occurring nucleotide sequence (e.g. encoding the original amino acid sequence of the propre- or pro-cutinase produced by Fusarium solani pisi);

(b) chemically synthesized nucleotide sequences consisting of codons that are preferred by the new host and a nucleotide sequence resulting in stable messenger RNA in the new host, still encoding the original amino acid sequence;

(c) genetically engineered nucleotide sequences derived from one of the nucleotide sequences mentioned in preceding paragraphs a or b coding for a Fusarium solani pisi Cutinase with a different amino acid sequence but having superior stability and/or activity in detergent systems.

Summarizing, rDNA vectors able to direct the expression of the nucleotide sequence encoding a Cutinase gene as described above in one of the preferred hosts preferably comprise the following components:

(a) Double-stranded (ds) DNA coding for mature Cutinase or precutinase or a corresponding precutinase in which at least part of the presequence has been removed directly down stream of a secretion signal (preferred for the selected host cell). In cases where the part of the gene that should be translated does not start with the codon ATG, an ATG codon should be placed in front. The translated part of the gene should always end with an appropriate stop codon;

(b) An expression regulon (suitable for the selected host organism) situated upstream of the plus strand of the ds DNA encoding the Cutinase (component (a));

(c) A terminator sequence (suitable for the selected host organism) situated down stream of the plus strand of the ds DNA encoding the Cutinase (component (a);

(d1) Nucleotide sequences which facilitate integration, of the ds DNA into the genome of the selected host or,

(d2) an origin of replication suitable for the selected host;

(e1) Optionally a (auxotrophic) selection marker. The auxotrophic marker can consist of a coding region of the auxotrophic marker and a defective promoter;

(e2) Optionally a ds DNA sequence encoding proteins involved in the maturation and/or secretion of one of the precursor forms of the Cutinase in the host selected.

Such a rDNA vector can also carry, upstream and/or downstream of the polynucleotide as earlier defined, further sequences facilitative of functional expression of the cutinase. The auxotrophic marker can consist of a coding region of the auxotrophic marker and a defective promoter region.

Another embodiment of this invention is the fermentative production of one of the various Cutinase variants described above. Such a fermentation can either be a normal batch fermentation, fed-batch fermentation or continuous fermentation. The selection of a process to be used depends on the host strain and the preferred down stream processing method (known per se). Thus, the invention also provides a process for producing a Cutinase variant as specified herein, which comprises the steps of fermentatively cultivating an rDNA modified micro-organism containing a gene made by rDNA technique which carries at least one mutation affecting the amino acid sequence of the Cutinase thereby to confer upon the Cutinase improved activity by comparison with the corresponding parent enzyme, making a preparation of the Cutinase variant by separating the Cutinase produced by the micro-organism either from the fermentation broth, or by separating the cells of the micro-organism from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the Cutinase variant either from said broth or from said cells by physical or chemical concentration or purification methods. Preferably conditions are chosen such that the Cutinase variant is secreted by the micro-organism into the fermentation broth, the enzyme being recovered from the broth after removal of the cells either by filtration or centrifugation. Optionally, the Cutinase variant can then be concentrated and purified to a desired extent. These fermentation processes in themselves apart from the special nature of the micro-organisms can be based on known fermentation techniques and commonly used fermentation and down stream processing equipment.

Also provided by the invention is a method for the production of a modified micro-organism capable of producing a Cutinase variant by means of rDNA techniques, characterized in that the gene coding for the Cutinase variant that is introduced into the micro-organism is fused at its 5'-end to a gene fragment encoding a (modified) pre-sequence functional as a signal- or secretion-sequence for the host organism.

According to a further aspect of the invention, there are provided rDNA modified micro-organisms containing a Cutinase variant gene and able to produce the Cutinase variant encoded by said gene. In an rDNA modified micro-organism, a gene (if originally present) encoding the native Cutinase is preferably removed, e.g. replaced by another structural gene.

According to a further aspect of the present invention, there are provided enzymatic detergent compositions comprising the Cutinase variants of the invention. Such compositions are combinations of the Cutinases variants and other ingredients which are commonly used in detergent systems, including additives for detergent compositions and fully-formulated detergent and cleaning compositions, e.g. of the kinds known per se and described for example in EP-A-258 068. More specifically, they may comprise from 5 - 60, preferably from 20 - 50% by weight of a detergency builder and from 0.1 - 50 % by weight of an active system, which in turn comprises 0 - 95 % by weight of one or more anionic surfactants and 5 - 100 % by weight of one or more nonionic surfactants.

The other components of such detergent compositions can be of any of many known kinds, for example as described in GB-A-1 372 034 (Unilever), US-A-3 950 277, US-A-4 011 169, EP-A-179 533 (Procter & Gamble), EP-A-205 208 and EP-A-206 390 (Unilever), JP-A-63-078000 (1988), and Research Disclosure 29056 of June 1988, together with each of the several specifications mentioned therein, all of which are hereby incorporated herein by reference.

The Cutinase variants of the present invention can usefully be added to the detergent composition in any suitable form, i.e. the form of a granular composition, a solution or a slurry of the enzyme, or with carrier material (e.g. as in EP-A-258 068 and the Savinase(TM) and Lipolase(TM) products of Novo Nordisk).

The added amount of Cutinase variant can be chosen within wide limits, for example from 10 - 20,000 LU per gram, and preferably 50 -2,000 LU per gram of the detergent

composition. In this specification LU or lipase units are defined as they are in EP-A-258 068 (Novo Nordisk).

Similar considerations apply mutatis mutandis in the case of other enzymes, which may also be present. See also European patent application EP-A-407 225.

Advantage may be gained in such detergent compositions, where protease is present together with the cutinase, by selecting such protease from those having pI lower than 10. EP-A-271 154 (Unilever) describes a number of such proteases. Proteases for use together with Cutinase variants can in certain circumstances include subtilisin of for example BPN' type or of many of the types of subtilisin disclosed in the literature, some of which have already been proposed for detergents use, e.g. mutant proteases as described in for example EP-A-130 756 or EP-A-251 446 (both Genentech); US-A-4 760 025 (Genencor); EP-A-214 435 (Henkel); WO-A-87/04661 (Amgen); WO-A-87/05050 (Genex); Thomas et al. (1986/5) Nature 316, 375-376 and (1987) J.Mol.Biol. 193, 803-813; Russel et al. (1987) Nature 328, 496-500.

The invention will now be further illustrated in the following Examples. All techniques used for the manipulation and analysis of nucleic acid materials were performed essentially as described in Sambrook et al. (1989), except where indicated otherwise.

In the accompanying drawings is:

Fig. 1A. Nucleotide sequence of cassette 1 of the synthetic Fusarium solani pisi cutinase gene and of the constituting oligo-nucleotides. Oligonucleotide transitions are indicated in the cassette sequence. Lower case letters refer to nucleotide positions outside the open reading frame.

Fig. 1B. Nucleotide sequence of cassette 2 of the synthetic Fusarium solani pisi cutinase gene and of the constituting oligo-nucleotides. Oligonucleotide transitions are indicated in the cassette sequence.

Fig. 1C. Nucleotide sequence of cassette 3 of the synthetic Fusarium solani pisi cutinase gene and of the

constituting oligo-nucleotides. Oligonucleotide transitions are indicated in the cassette sequence. Lower case letters refer to nucleotide positions outside the open reading frame.

Fig. 1D. Nucleotide sequence of the synthetic cutinase gene encoding Fusarium solani pisi pre-pro-cutinase. The cutinase pre-sequence, pro-sequence and mature sequence are indicated. Also the sites used for cloning and the oligonucleotide transitions are indicated. Lower case letters refer to nucleotide positions outside the open reading frame.

Fig. 2. Nucleotide sequence of a synthetic DNA fragment for linking the Fusarium solani pisi pro-cutinase encoding sequence to a sequence encoding a derivative of the E. coli phoA pre-sequence. The ribosome binding site (RBS) and the restriction enzyme sites used for cloning are indicated. Also the amino acid sequences of the encoded phoA signal sequence and part of the cutinase gene are indicated using the one-letter code.

Fig. 3. Nucleotide sequence of cassette 8, a SacI-BclI fragment which encodes the fusion point of the coding sequences for the invertase pre-sequence and mature Fusarium solani pisi cutinase.

Fig. 4. Plasmid pUR2741 obtained by deletion of a 0.2 kb SalI-NruI from pUR2740, is an E. coli-S. cerevisiae shuttle vector comprising part of pBR322, an origin of replication in yeast cells derived from the 2μm plasmid, a yeast leu2D gene and a fusion of the yeast invertase signal sequence encoding region with a plant α-galactosidase gene under the control of the yeast gal7 promoter.

Fig. 5. Plasmid pUR7219 is an E. coli-S. cerevisiae shuttle vector comprising part of pBR322, an origin of replication in yeast cells derived from the 2μm plasmid, a yeast leu2D gene and a fusion of the yeast invertase signal sequence encoding region with the region encoding the mature Fusarium solani

pisi cutinase under the control of the yeast gal7 promoter.

Fig. 6. Plasmid pUR2740 is an E. coli-S. cerevisiae shuttle vector comprising part of pBR322, an origin of replication in yeast cells derived from the 2μm plasmid, a yeast leu2D gene and a fusion of the yeast invertase signal sequence encoding region with a plant α-galactosidase gene under the control of the yeast gal7 promoter.

Fig. 7. Nucleotide sequence of cassettes 5, 6 and 7, comprising different types of connections of the coding sequences of the exlA pre-sequence and mature Fusarium solani pisi cutinase.

Fig. 8. Plasmid pAW14B obtained by insertion of a 5.3 kb SalI fragment of Aspergillus niger var. awamori genomic DNA in the SalI site of pUC19.

Fig. 9. Plasmid pUR7280 obtained by displacing the BspHI-AflII fragment comprising the exlA open reading frame in pAW14B with a BspHI-AflII fragment comprising the Fusarium solani pisi pre-pro-cutinase coding sequence. Thus, plasmid pUR7280 comprises the Fusarium solani pisi pre-pro-cutinase gene under the control of the A. niger var. awamori promoter and terminator.

Fig. 10. Plasmid pUR7281 obtained by introduction of both the A. nidulans amdS and A. niger var. awamori pyrG selection markers in pUR7280.

Fig. 11. Schematical representation of the Fusarium solani pisi cutinase molecule.

Fig. 12. Partial amino acid sequences of the cutinases from Fusarium solani pisi, Colletotrichum capsici, Colletotrichum gloeosporiodes and Magnaporthe grisea, showing the location of the residues in the 3-D structure.

Fig. 13. In-the-wash effect for Fusarium solani pisi cutinase and the Cutinase variant N172K.

Fig. 14. In-the-wash effect for Fusarium solani pisi cutinase and the Cutinase variant E201K.

Fig. 15. In-the-wash effect for Fusarium solani pisi cutinase and the Cutinase variant A85F.

REFERENCES

Sambrook, J., Fritsch, E.F. and Maniatis,T. (1989). Molecular Cloning: a laboratory manual (2nd ed). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. ISNB 0-87969-309-6.

Fürste, J.P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P. Bagdasarian, M. and Lanka, E. (1986). Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector. Gene 48, 119-131.

Michaelis et al. (1983). J. Bacteriol 154, 366-

Tartof and Hobbs (1988). Gene 67, 169-182.

Soliday, C.L., Flurkey, W.H, Okita, T.W. and Kolattukudy, P.E. (1984). Cloning and structure determination of cDNA for cutinase, an enzyme involved in fungal penetration of plants. Proc.Natl.Acad.Sci. USA 81, 3939-3943.

Nogi, Y. and Fukasawa, T. (1983). Nucleotide sequence of the transcriptional initiation region of the yeast GAL7 gene. Nucleic Acids Res. 11, 8555-8568.

Taussig, R. and Carlsson, M. (1983). Nucleotide sequence of the yeast SUC2 gene for invertase. Nucleic Acids Res. 11, 1943-1954.

Erhart, E and Hollenberg, C.P. (1981) Curing of Saccharomyces cerevisiae 2-μm DNA by transformation. Curr. Genet. 3, 83-89.

Verbakel, J.A.M.V. (1991) Heterologous gene expression in the yeast Saccharomyces cerevisiae. Ph.D. thesis. Rijks Universiteit Utrecht, The Netherlands.

Maat, J., Roza, M., Verbakel, J., Stam, J., Santos da Silva, M.J., Bosse, M., Egmond, M.R., Hagemans, M.L.D., v. Gorcom, R.F.M., Hessing, J.G.M., v.d. Hondel, C.A.M.J.J. and v. Rotterdam, C. (1992). Xylanases and their application in bakery. In: Visser, J., Beldman, G., Kusters-van Someren, M.A. and Voragen, A.G.J. (Eds), Xylans and Xylanases. Progress in Biotechnology Volume 7, Elsevier Science Publishers, Amsterdam. ISBN 0-444-89477-2.

de Graaff, L.H., van den Broek, H.C., van Ooijen, A.J.J. and Visser, J. (1992). Structure and regulation of an Aspergillus xylanase gene. In: Visser, J., Beldman, G., Kusters-van Someren, M.A. and Voragen, A.G.J. (Eds), Xylans and Xylanases. Progress in Biotechnology Volume 7, Elsevier Science Publishers, Amsterdam. ISBN 0-444-89477-2.

Hankin, L. and Kolattukudy, P.E. (1968). J. Gen. Microbiol. 51, 457-463.

Ettinger, W.F., Thukral, S.K. and Kolattukudy, P.E. (1987). Structure of cutinase gene, cDNA, and the derived amino acid sequence from phytopathogenic fungi. Biochemistry 26, 7883-7892.

Huse, W.D. and Hansen, C. (1988). Strategies 1, 1-3.

De Graaff, L.H., H.W.J. van den Broek and J. Visser (1988). Isolation and expression of the Aspergillus nidulans pyruvate kinase gene. Curr. Genet. 13, 315-321.

EXAMPLE 1

Construction of a synthetic gene encoding Fusarium solani pisi pre-pro-cutinase.

A synthetic gene encoding Fusarium solani pisi pre-pro-cutinase was constructed essentially according to the method described in EP-A-407 225 (Unilever). Based on published nucleotide sequences of Fusarium solani pisi genes (Soliday et al. (1984) and WO-A-90/09446, Plant Genetic Systems), a completely synthetic DNA fragment was designed which comprises a region encoding the Fusarium solani pisi pre-pro-cutinase polypeptide. Compared to the nucleotide sequence of the original Fusarium solani pisi gene, this synthetic cutinase gene comprises several nucleotide changes through which restriction enzyme recognition sites were introduced at convenient positions within the gene without affecting the encoded amino acid sequence. The nucleotide sequence of the entire synthetic cutinase gene is presented in Fig. 1D.

Construction of the synthetic cutinase gene was performed by assembly of three separate cassettes starting

from synthetic DNA oligonucleotides. Each synthetic DNA cassette is equipped with an EcoRI site at the start and a HindIII site at the end. Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis. For the construction of each of the cassettes the procedure outlined below was followed. Equimolar amounts (50 pmol) of the oligonucleotides constituting a given cassette were mixed, phosphorylated at their 5'-end, annealed and ligated according to standard techniques. The resulting mixture of double stranded DNA molecules was cut with EcoRI and HindIII, size-fractionated by agarose gel electrophoresis and recovered from the gel by electro-elution. The resulting synthetic DNA cassette was ligated with the 2.7 kb EcoRI-HindIII fragment of pUC9 and transformed to Escherichia coli. The EcoRI-HindIII insert of a number of clones was completely sequenced in both directions using suitable oligonucleotide primers to verify the sequence of the synthetic cassettes. Using this procedure pUR7207 (comprising cassette 1, Fig. 1A), pUR7208 (comprising cassette 2, Fig. 1B) and pUR7209 (comprising cassette 3, Fig. 1C) were constructed. Finally, the synthetic cutinase gene was assembled by combining the 2.9 kb EcoRI-ApaI fragment of pUR7207 with the 0.2 kb ApaI-NheI fragment of pUR7208 and the 0.3 kb NheI-HindIII fragment of pUR7209, yielding pUR7210. This plasmid comprises an open reading frame encoding the complete pre-pro-cutinase of Fusarium solani pisi (Fig. 1D).

EXAMPLE 2

Expression of Fusarium solani pisi (pro)cutinase in Escherichia coli.

With the synthetic cutinase gene an expression vector for E. coli was constructed which is functionally similar to the one described in WO-A-90/09446 (Plant Genetic Systems). A construct was designed in which the part of the synthetic gene encoding Fusarium solani pisi pro-cutinase is preceded by proper E. coli expression signals, i.e. (i) an inducible promoter, (ii) a ribosome binding site and (iii) a signal sequence which provides a translational initiation

codon and provides information required for the export of the pro-cutinase across the cytoplasmic membrane.

A synthetic linker was designed (see Fig. 2) for fusion of a derivative of the E. coli phoA signal sequence (Michaelis et al., 1983) to the pro-sequence of the synthetic cutinase gene. To optimize cleavage of the signal peptide and secretion of pro-cutinase, the nucleotide sequence of this linker was such that the three C-terminal amino acid residues of the phoA signal sequence (Thr-Lys-Ala) were changed into Ala-Asn-Ala and the N-terminal amino acid residu of the cutinase pro-sequence (Leu 1, see Fig. 1D) was changed into Ala. This construction ensures secretion of cutinase into the periplasmatic space (see WO-A-90/09446, Plant Genetic Systems).

To obtain such a construct, the 69 bp EcoRI-SpeI fragment comprising the cutinase pre-sequence and part of the pro-sequence was removed from pUR7210 and replaced with the synthetic DNA linker sequence (EcoRI-SpeI fragment) providing the derivative of the E. coli phoA pre-sequence and the altered N-terminal amino acid residue of the cutinase pro-sequence (Fig. 2). The resulting plasmid was named pUR7250 and was used for the isolation of a 0.7 kb BamHI-HindIII fragment comprising a ribosome binding site and the pro-cutinase encoding region fused to the phoA signal sequence encoding region. This fragment was ligated with the 8.9 kb BamHI-HindIII fragment of pMMB67EH (Fürste et al., 1986) to yield pUR7220. In this plasmid the synthetic gene encoding pro-cutinase is fused to the altered version of the phoA signal sequence and placed under the control of the inducible tac-promoter.

E. coli strain WK6 harboring pUR7220 was grown in 2 litre shakeflasks containing 0.5 litre IXTB medium (Tartof and Hobbs, 1988) consisting of:

0.017 M KH_2PO_4
0.017 M K_2HPO_4
12 g/l Bacto-tryptone
24 g/l Bacto-yeast extract
0.4 % glycerol (v/v)

Cultures were grown overnight at 25°C - 30°C in the presence of 100 μg/ml ampicillin under vigorous shaking (150 rpm) to an OD at 610 nm of 10-12. Then IPTG (isopropyl-ß-D-thiogalactopyranoside) was added to a final concentration of 10 μM and incubation continued for another 12-16 hours. When no further significant increase in the amount of produced lipolytic activity could be observed, as judged by the analysis of samples withdrawn from the cultures, the cells were harvested by centrifugation and resuspended in the original culture volume of buffer containing 20% sucrose at 0°C. The cells were collected by centrifugation and resuspended in the original culture volume of icecold water causing lysis of the cells through osmotic shock. Cell debris was removed by centrifugation and the cell free extract was acidified to pH 4.8 with acetic acid, left overnight at 4°C and the resulting precipitate was removed. A better than 75% pure cutinase preparation essentially free of endogenous lipases was obtained at this stage by means of ultra-filtration and freeze drying of the cell free extract. Alternatively, cutinase could be purified to homogeneity (i.e. better than 95% pure) by loading the acidified cell free extract onto SP-sephadex, eluting the enzyme with buffer at pH 8.0, passage of the concentrated alkaline solution through a suitable volume of DEAE-cellulose (Whatman DE-52) and direct application of the DEAE flow-through to a Q-sepharose HP (Pharmacia) column. Elution with a salt gradient yielded a homogenous cutinase preparation with a typical overall yield of better than 75%.

EXAMPLE 3

Construction of genes encoding variants of Fusarium solani pisi cutinase.

Using the synthetic gene for Fusarium solani pisi pre-pro-cutinase described in Example 1, variant genes comprising alterations in the encoded amino acid sequence were constructed. For this construction essentially the same approach was followed as described in Example 1 for the construction of the three cassettes constituting the complete

synthetic cutinase gene. For example, a gene coding for Fusarium solani pisi cutinase variant N172K was constructed by assembling a new version of cassette 3 using the same oligonucleotides as described in Example 1, except for the two oligos which cover the coding triplet for the position which is to be mutated, i.c. Asn 172. Instead, two new synthetic oligos were used, which comprise the mutant sequence but are otherwise identical to the original oligos which they are replacing. More specifically, a new cassette 3 comprising mutation N172K was assembled by incorporating a variant of CUTI3D MH (containing AAG instead of AAT) and a variant of CUTI3K MH (containing CTT instead of ATT) instead of CUTI3D MH and CUTI3K MH (see Fig. 1C). The new cassette 3 was cloned and sequenced essentially as described in Example 1 and the 0.3 kb NheI-HindIII DNA fragment comprising the mutation was exchanged for the corresponding fragment in pUR7210, yielding pUR7257 (N172K). The 0.6 kb SpeI-HindIII fragment from this plasmids was used to replace the corresponding fragment in pUR7220, yielding the E. coli expression plasmid pUR7224 (N172K). This E. coli expression plasmid was transformed to E. coli strain WK6. Transformants were grown as outlined in Example 2 and the variant pro-cutinase enzyme was recovered and purified essentially as described in Example 2.

A gene coding for Fusarium solani pisi cutinase variant E201K was constructed in an analogous way by assembling a new version of cassette 3 incorporating a variant of CUTI3F MH (containing AAG instead of GAG) and a variant of CUTI3M MH (containing CTT instead of CTC) instead of CUTI3F MH and CUTI3M MH (see Fig. 1C).

A gene coding for Fusarium solani pisi cutinase variant A85F was constructed in an analogous way by assembling a new version of cassette 2 incorporating a variant of CUTI2C MH (containing TTC instead of GCT) and a variant of CUTI2I MH (containing GAA instead of AGC) instead of CUTI2C MH and CUTI2I MH (see Fig. 1B).

EXAMPLE 4

Expression of Fusarium solani pisi cutinase in Saccharomyces cerevisiae.

For the expression of the synthetic Fusarium solani pisi cutinase gene in Saccharomyces cerevisiae an expression vector was constructed in which a synthetic gene encoding the mature cutinase is preceded by the pre-sequence of S. cerevisiae invertase (Taussig and Carlsson, 1983) and the strong, inducible gal7 promoter (Nogi and Fukasawa, 1983). To prepare the synthetic cutinase gene for such a fusion, an adaptor fragment was synthetized in which the coding sequence for the invertase pre-sequence is fused to the sequence encoding the N-terminus of mature cutinase. This fragment was assembled as an EcoRI-HindIII cassette in pUC9 essentially as described in Example 1 (cassette 8, see Fig. 3), yielding pUR7217. Plasmids pUR7210 and pUR7217 were transformed to E. coli JM110 (a strain lacking the dam methylase activity) and the 2.8 kb BclI-HindIII fragment of pUR7217 was ligated with the 0.6 kb BclI-HindIII fragment of pUR7210, yielding pUR7218 in which the nucleotide sequence coding for the mature cutinase polypeptide is fused with part of the S. cerevisiae invertase pre-sequence coding region.

The expression vector pUR2741 (see Fig. 4) was derived from pUR2740 (Verbakel, 1991, see Fig. 6) by isolation of the 8.9 kb NruI-SalI fragment of pUR2740, filling in the SalI protruding end with Klenow polymerase, and recircularization of the fragment. The 7.3 kb SacI-HindIII fragment of pUR2741 was ligated with the 0.7 kb SacI-HindIII fragment of pUR7218, yielding pUR7219 (see Fig. 5). Optionally, a S. cerevisiae polII terminator can be placed behind the cutinase gene, in the HindIII site, which turned out not to be essential for efficient expression of the cutinase gene. The E. coli-S. cerevisiae shuttle plasmid pUR7219 contains a origin for replication in S. cerevisiae strains harboring the 2μ plasmid (cir^+ strains), a promoter-deficient version of the S. cerevisiae Leu2 gene permitting selection of high copy number transformants in S. cerevisiae $leu2^-$ strains, and the synthetic gene encoding the mature

part of Fusarium solani pisi cutinase operably linked to the S. cerevisiae invertase pre-sequence under the regulation of the strong, inducible S. cerevisiae gal7 promoter.

S. cerevisiae strain SU50 (a, cir^0, leu2, his4, can1), which is identical to strain YT6-2-1L (Erhart and Hollenberg, 1981), was co-transformed with an equimolar mixture of the 2μ S. cerevisiae plasmid and pUR7219 using a standard protocol for electroporation of yeast cells. Transformants were selected for leucine prototrophy and total DNA was isolated from a number of transformants. All transformants appeared to contain both the 2μ plasmid and pUR7219, exemplifying that the promoter-deficient version of the leu2 gene contained on pUR7219 can only functionally complement leu2 deficient strains when present in high copy numbers due to the simultaneous presence of the 2μ yeast plasmid. One of the transformants was cured for the pUR7219 plasmid by cultivation on complete medium for more than 40 generations followed by replica-plating on selective and complete solid media, yielding S. cerevisiae strain SU51 (a, cir$^+$, leu2, his4, can1).

S. cerevisiae strain SU51 harboring pUR7219 was grown in 1 litre shakeflasks containing 0.2 litre MM medium consisting of:

- yeast nitrogen base (YNB) without amino acids	6.7	g/l
- histidine	20	mg/l
- glucose	20	g/l

Cultures were grown overnight at 30°C under vigorous shaking (150 rpm) to an OD at 610 nm of 2-4. Cells were collected by centrifugation and resuspended in 1 litre of YPGAL medium consisting of:

- yeast extract	10	g/l
- bacto peptone	20	g/l
- galactose	50	g/l

in 2 litre shake flasks and incubation continued for another 12-16 hours. At regular intervals samples were withdrawn from the culture and centrifugated to remove biomass. The supernatant was analyzed for cutinase activity by a titrimatic assay using olive oil as a substrate. For each

sample between 100 and 200 µl of filtrate was added to a stirred mixture of 5.0 ml lipase substrate (Sigma, containing olive oil as a substrate for the lipase) and 25.0 ml of buffer (5 mM Tris-HCl pH 9.0, 40 mM NaCl, 20 mM $CaCl_2$). The assay was carried out at 30°C and the release of fatty acids was measured by automated titration with 0.05 M NaOH to pH 9.0 using a Mettler DL25 titrator. A curve of the amount of titrant against time was obtained. The amount of lipase activity contained in the sample was calculated from the maximum slope of this curve. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol of fatty acid from olive oil in one minute under the conditions specified above. Such determinations are known to those skilled in the art.

When the production of cutinase activity did no longer increase, cells were removed by centrifugation and the cell free extract was acidified to pH 4.8 with acetic acid and cutinase was recovered as described in Example 1.

EXAMPLE 5

Expression of variants of Fusarium solani pisi cutinase in S. cerevisiae.

Variant N176K of Fusarium solani pisi cutinase was expressed in S. cerevisiae in the following way. The 0.5 kb ApaI-HindIII fragment of pUR7257 (N172K) was used to replace the analogous fragment of pUR7218, yielding pUR7228 (N172K), in which the gene comprising the mutation is operably fused to the sequence encoding the S. cerevisiae signal sequence. The 7.0 kb SacI-HindIII fragment of pUR2741 was ligated with the 0.7 kb SacI-HindIII fragment of pUR7228 (N172K), yielding pUR7234 (N172K). This plasmid was transformed to S. cerevisiae strain SU51. The resulting transformants were incubated as described in Example 4 and the variant enzyme produced was recovered from the culture broth as described in Examples 4 and 1.

Variant E201K of Fusarium solani pisi cutinase was produced in S. cerevisiae in the same way 5 using a variant

of the Fusarium solani pisi cutinase gene coding for the cutinase variant E201K, as described in Example 3.

Variant A85F of Fusarium solani pisi cutinase was produced in S. cerevisiae in the same way using a variant of the Fusarium solani pisi cutinase gene coding for the cutinase variant A85F, as described in Example 3.

EXAMPLE 6

Expression of Fusarium solani pisi cutinase in Aspergilli.

For the expression of the synthetic Fusarium solani pisi cutinase gene in Aspergillus niger var. awamori an expression vector was constructed in which the synthetic gene encoding Fusarium solani pisi pre-pro-cutinase was placed under the control of the A. niger var. awamori strong, inducible exlA promoter (Maat et al.,1992, de Graaff et al., 1992).

The pre-pro-cutinase expression plasmid (pUR7280) was constructed starting from plasmid pAW14B, which was deposited in an E. coli strain JM109 with the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, under N° CBS 237.90 on 31st May 1990, and contains a ca. 5.3 kb SalI fragment on which the 0.7 kb endoxylanase II (exlA) gene is located, together with 2.5 kb of 5'-flanking sequences and 2.0 kb of 3'-flanking sequences (Fig.8). In pAW14B the exlA coding region was replaced by the pre-pro-cutinase coding region. A BspHI site (5'-TCATGA-3') comprising the first codon (ATG) of the exlA gene and an AflII site (5'-CTTAAG-3'), comprising the stopcodon (TAA) of the exlA gene facilitated the construction of pUR7280.

The construction was carried out as follows: pAW14B (7.9 kb) was cut partially with BspHI and the linearized plasmid (7.9 kb) was isolated from an agarose gel. Subsequently, the isolated 7.9 kb fragment was cut with BsmI, which cuts a few nucleotides downstream of the BspHI site of interest, to remove plasmids linearized at other BspHI sites. The fragments were separated on an agarose gel and the 7.9 kb BspHI-BsmI fragment was isolated. This was partially cut with

AflII and the resulting 7.2 kb BspHI-AflII fragment was isolated.

The 0.7 kb BspHI-AflII fragment of pUR7210 comprising the entire open reading frame coding for Fusarium solani pisi pre-pro-cutinase was ligated with the 7.2 kb BspHI-AflII fragment of pAW14B, yielding pUR7280. The constructed vector (pUR7280) can subsequently transferred to moulds (for example Aspergillus niger, Aspergillus niger var. awamori, etc) by means of conventional co-transformation techniques and the pre-pro-cutinase gene can then be expressed via induction of the endoxylanaseII promoter. The constructed rDNA vector can also be provided with conventional selection markers (e.g. amdS or pyrG, hygromycin etc.) and moulds can be transformed with the resulting rDNA vector to produce the desired protein. As an example, the amdS and pyrG selection markers were introduced in the expression vector, yielding pUR7281 (Fig. 10). For this purpose a NotI site was created by converting the EcoRI site (present 1.2 kb upstream of the ATG codon of the pre-pro-cutinase gene) into a NotI site using a synthetic oligonucleotide (5'-AATTGCGGCCGC-3'), yielding pUR7282. Suitable DNA fragment comprising the entire A. nidulans amdS gene and the A. niger var. awamori pyrG gene together with their own promoters and terminators were equiped with flanking NotI sites and introduced in the NotI site of pUR7282, yielding pUR7281 (Fig. 10).

As an alternative approach for the expression of the synthetic Fusarium solani pisi cutinase gene in Aspergillus niger var. awamori, expression vectors were constructed in which a synthetic gene encoding the mature cutinase is not preceded by its own pre-pro-sequence, but by the pre-sequence of A. niger var. awamori exlA.

To prepare the synthetic cutinase gene for such fusions, several adaptor fragments were synthetized in which the coding sequence for the exlA pre-sequence is connected to the sequence encoding the N-terminus of mature cutinase in different ways. In cassette 5 this connection is made by fusing the exlA pre-sequence to the pro-sequence of cutinase.

In cassette 6 the exlA pre-sequence is fused with the N-terminal residue of mature cutinase. Cassette 7 is identical with cassette 6, but here the N-terminal residue of the encoded mature cutinase polypeptide has been changed from the original Glycine into a Serine residue in order to better fit the requirements for cleavage of the signal peptide. Cassettes 5, 6 and 7 were assembled from synthetic oligonucleotides essentially as described in Example 1 (see Fig. 7). Cassette 5 was used to displace the 0.1 kb EcoRI-SpeI fragment of pUR7210, yielding pUR7287. Cassettes 6 and 7 were used to displace the 0.1 kb EcoRI-BclI fragment of pUR7210, yielding pUR7288 and pUR7289, respectively. For each of the plasmids pUR7287, pUR7288 and pUR7289 the 0.7 kb BspHI-AflII fragment was ligated with the 7.2 kb BspHI-AflII fragment of pAW14B, yielding pUR7290, pUR7291 and pUR7292, respectively.

The constructed rDNA vectors subsequently were transferred to moulds (Aspergillus niger, Aspergillus niger var. awamori) by means of conventional co-transformation techniques and the pre-(pro)-cutinase gene were expressed via induction of the endoxylanaseII promoter. The constructed rDNA vectors can also be provided with conventional selection markers (e.g. amdS or pyrG, hygromycin) and the mould can be transformed with the resulting rDNA vector to produce the desired protein, as illustrated in this example for pUR7280 (see above).

Aspergillus strains transformed with either of the expression vectors pUR7280, pUR7281, pUR7290, pUR7291, pUR7292 (containing the Fusarium solani pisi mature cutinase encoding region with or without the corresponding pro-sequence and either the cutinase signal sequence or the exlA signal sequence under the control of A. niger var. awamori exlA promoter and terminator) were grown under the following conditions: multiple 1 litre shake flasks with 400 ml synthetic media (pH 6.5) were inoculated with spores (final concentration: 10E6/ml). The medium had the following composition (AW Medium):

sucrose	10	g/l
$NaNO_3$	6.0	g/l
KCl	0.52	g/l
KH_2PO_4	1.52	g/l
$MgSO_4 \cdot 7H_2O$	0.49	g/l
Yeast extract	1.0	g/l
$ZnSO_4 \cdot 7H_2O$	22	mg/l
H_3BO_3	11	mg/l
$MnCl_2 \cdot 4H_2O$	5	mg/l
$FeSO_4 \cdot 7H_2O$	5	mg/l
$CaCl_2 \cdot 6H_2O$	1.7	mg/l
$CuSO_4 \cdot 5H_2O$	1.6	mg/l
$NaH_2MoO_4 \cdot 2H_2O$	1.5	mg/l
Na_2EDTA	50	mg/l

Incubation took place at 30°C, at 200 rpm for 24 hours in a Mk X incubator shaker. After growth cells were collected by filtration (0.45 μm filter), washed twice with AW Medium without sucrose and yeast extract (salt solution), resuspended in 50 ml salt solution and transferred to 300 ml shake flasks containing 50 ml salt solution to which xylose has been added to a final concentration of 10 g/l (induction medium). Incubation under the same conditions as described above was continued overnight. The resulting cultures were filtered over miracloth to remove biomass and cutinase was recovered essentially as described in Example 2.

EXAMPLE 7

Expression of variants of Fusarium solani pisi cutinase in Aspergilli.

By following essentially the route outlined in Example 6, but now using plasmid pUR7257 (N172K) instead of pUR7210 for the construction of fungal expression vectors, a variant of Fusarium solani pisi cutinase comprising mutation N172K was produced in Aspergillus niger var. awamori.

EXAMPLE 8

Identification and isolation of genes related to the Fusarium solani pisi cutinase gene.

Genes encoding cutinases with a varying degree of homology with Fusarium solani pisi cutinase were isolated from different fungi. Fungal cultures were grown in 500 ml shakeflasks containing 200 ml of the medium described by Hankin and Kolattukudy (1968) supplemented with 0.25% glucose and incubated for 4 days at 28°C in a Mk X incubator shaker (100 rpm). At this time the glucose had been consumed and cutinase production was induced by the addition of cutin hydrolysate essentially as described by Ettinger et al. (1987). At regular intervals samples were withdrawn from the culture and analyzed for the presence of lipolytic activity according to standard techniques (see example 4). Normally, about two days after induction lipolytic activity could be demonstrated and at that time the cells were harvested by filtration using standard techniques. The mycelia were washed, frozen in liquid nitrogen and lyophilized according to standard techniques. Total cellular RNA preparations were isolated using the guanidinium thiocyanate method and purified by cesium chloride density gradient centrifugation, essentially as described by Sambrook et al. (1989). PolyA(+) mRNA fractions were isolated using a polyAT tract mRNA isolation kit (Promga). The polyA(+) mRNA fractions were used in a Northern hybridization analysis using a cDNA fragment from the Fusarium solani pisi cutinase gene as a probe according to standard techniques, to verify the expression of cutinase-related genes. Preparations of mRNA comprising material capable of hybridizing with the probe were used for the synthesis of cDNA using a ZAP cDNA synthesis kit (Stratagene, La Jolla) according to the instructions of the supplier, yielding cDNA fragments with an XhoI cohesive end flanking the poly-A region and an EcoRI adaptor at the other end. The obtained cDNA fragments were used for the construction of expression libraries by directional cloning in the sense orientation in lambda ZAPII vectors (Stratagene, La Jolla), allowing expression of ß-galactosidase fusion proteins (Huse et al.,1988). These libraries were screened using antiserum raised against Fusarium solani pisi cutinase.

Alternatively, the synthesized cDNA fractions were subjected to PCR-screening using cutinase specific primers (see table 2). These primers were derived from comparison of the amino acid sequence of several fungal Cutinase genes (Ettinger et al., 1987). The conditions for the PCR reaction were optimized for each set of primers, using cDNA from Fusarium solani pisi cutinase as a control. For those preparations of cDNA with which a specific PCR fragment could be generated with a length that is similar to (or greater than) the length of the PCR fragment generated with the cDNA from Fusarium solani pisi cutinase under the same conditions, the PCR fragment was purified by gel electroforesis and isolated from the gel.

As an alternative approach, the PCR screening technique using cutinase specific primers was also applied directly to genomic DNA of some fungal strains, using genomic DNA of Fusarium solani pisi as a positive control. For those preparations of fungal genomic DNA with which a specific PCR fragment could be generated with a length that is similar to (or greater than) the length of the PCR fragment generated with the cDNA from Fusarium solani pisi cutinase under identical conditions, the PCR fragment was purified by gel electrophoresis and isolated from the gel.

For strains which scored positive in either the expression library approach or the PCR screening approach (either with cDNA or genomic DNA) as well as a number of other strains, high molecular weight genomic DNA was isolated. Strains were grown essentially as described by Ettinger et al. (1987), and genomic DNA was isolated as described by de Graaff et al. (1988). Genomic DNA was digested with various restriction enzymes and analyzed by Southern hybridization using either the analogous cDNA insert (expression library approach) or the PCR fragment (PCR screening approach) or the Fusarium solani pisi cutinase gene (other strains) as a probe, and a physical map of the cutinase genes was constructed. An appropriate digest of genomic DNA was size-fractionated by gel electrophoresis and fragments of the appropriate size were isolated from the gel

and subcloned in pUC19. These genomic libraries were screened with the corresponding cDNA insert (expression library approach) or the PCR fragment (PCR screening approach), yielding clones comprising the genomic copy of the cutinase genes. These genes were sequenced in both directions. Introns were identified by sequencing the corresponding cDNA or by comparison with other cutinase sequences (Ettinger et al., 1987). The N-terminal end of the mature cutinase polypeptide was also deduced from such a comparison. Using standard PCR techniques, the introns were removed, a HindIII site was engineered immediately downstream of the open reading frames and the coding sequence for the pre-sequence of the Saccharomyces cerevisiae invertase gene (preceded by a SacI site, compare cassette 8, Fig. 3) was fused to the sequences encoding the N-terminus of the mature cutinases. The obtained SacI-HindIII fragments comprising the cutinase genes operably linked to the sequence encoding the S. cerevisiae invertase pre-sequence were ligated with the 7.3 kb SacI-HindIII fragment of pUR7241 (see Fig. 4) and transformed to S. cerevisiae strain SU51. The fungal cutinases were expressed and recovered from the culture broth essentially as described in Example 4.

EXAMPLE 9

The In-the-wash activity of Fusarium solani pisi Cutinase variant N172K.

The effect on fat removal of the Cutinase variant N172K was compared to that of the wild type Fusarium solani pisi Cutinase. In the test polyester test cloths soiled with brominated olive oil were used as monitors. The amount of fat on the test cloth was determined by measuring the amount of bromine on the test cloth by means of X-ray fluorescence spectrometry (as described above).

The amount of enzymatic soil removal of the wild type Fusarium solani pisi Cutinase (WT) and N172K variant was determined at a dosage of 3 LU/ml under several experimental conditions:

Soil removal (%) WT	Soil removal (%) N172K	Temperature (°C)	Detergent Product	Water hardness (FH)
4.9	10.1	40	A (1 g/l)	6
1.6	6.7	40	B (2 g/l)	6
20.2	24.6	40	C (2 g/l)	27
13.2	13.4	40	B (1 g/l)	27
30.8	40.6	30	C (2 g/l)	27
17.4	22.4	30	B (2 g/l)	27

The compositions (in % by weight) of the Detergent Products A-C were as follows:

Product A

compound	weight %
nonionic surfactant C_{12}-C_{15}alcohol 10.5-13EO	9.5
Sodium sulphate	38.6
Sodium Carbonate	40.4
Sodium silicate (Na_2O:Si_2O = 2.4)	7.3
Water	4.2

Product B

compound	weight %
DOBS	6.4
Soap	1.7
Synperonic A7	3.0
Zeolite	43
Sokolan CP7	9.9

waterglass	1.2
Sodium CMC	0.77
Sodium carbonate	10.16
NaOH	2.6
Water	to 100%

Product C

compound	weight %
Coco-primary alkyl sulphate	5.2
Nonionic surfactant C_{12}-C_{15} alcohol 7 EO	5.2
Nonionic surfactant C_{12}-C_{15} alcohol 3 EO	6.6
Sodium silicate	0.45
Zeolite 4A	32.00
Sodium carbonate	11.52
Hardened Tallow soap	2.00

The enhancement of the in-the-wash performance (oily soil removal) relative to wild-type Fusarium solani pisi cutinase under various wash conditions is evident. Figure 13 shows the in-the-wash performance at various enzyme concentrations using 2 g/l Detergent Product C in a 30 minute wash at 30°C at 27°FH.

For comparison, the same experiments were also carried out with Lipolase (TM). Under all conditions, cutinase variant N172K was superior.

EXAMPLE 12

The In-the-wash activity of Fusarium solani pisi Cutinase variant E201K.

The effect on fat removal of the Cutinase variant E201K at various enzyme concentrations was compared to that of the wild type Fusarium solani pisi Cutinase using 2 g/l Detergent Product C (see Example 11) in a 30 minute wash at 30°C at 27°FH. In the test polyester test cloths soiled with brominated olive oil were used as monitors. The amount of fat on the test cloth was determined by measuring the amount of bromine on the test cloth by means of X-ray fluorescence spectrometry (as described above).

The results are shown in Figure 14. The enhancement of the in-the-wash performance (oily soil removal) relative to wild-type Fusarium solani pisi cutinase is evident. For comparison, the same experiment was also carried out with Lipolase (TM). It was found that the performance of the E201K cutinase variant was clearly superior.

EXAMPLE 13

The In-the-wash activity of Fusarium solani pisi Cutinase variant A85F.

The effect on fat removal of the Cutinase variant A85F at various enzyme concentrations was compared to that of the wild type Fusarium solani pisi Cutinase using 2 g/l Detergent Product C (see Example 11) in a 30 minute wash at 30°C at 27°FH. In the test polyester test cloths soiled with brominated olive oil were used as monitors. The amount of fat on the test cloth was determined by measuring the amount of bromine on the test cloth by means of X-ray fluorescence spectrometry (as described above).

The results are shown in Figure 15. The enhancement of the in-the-wash performance (oily soil removal) relative to wild-type Fusarium solani pisi cutinase is evident. For comparison, the same experiment was also carried out with Lipolase (TM). It was found that the performance of the A85F cutinase variant was clearly superior.

*****

CLAIMS

1. A Cutinase variant of a parent Cutinase, wherein the amino acid sequence has been modified in such way that the hydrophobicity at the surface of the enzyme has been increased.

2. A Cutinase variant according to Claim 1, in which the hydrophobicity at the surface of the enzyme adjacent to the lipid contact zone has been increased so as to form an enlarged lipid contact zone.

3. A Cutinase variant according to any one of the preceding Claims, in which the hydrophobicity has been increased by replacing one or more amino acid residues by amino acid residues selected from the group consisting of valine, leucine, isoleucine, phenylalanine, tryptophan and methionine.

4. A Cutinase variant according to any of the preceding Claims, wherein the amino acid sequence has been modified in such way that in addition to the increase in hydrophobicity at the surface, one or more positive charges have been introduced.

5. A Cutinase variant according to Claim 4, in which the positive charges have been introduced by introduction of one or more lysine or arginine residues.

6. A Cutinase variant according to any one of the preceding Claims, in which the amino acid residue which is replaced has a small side chain, and is preferably selected from the group consisting of alanine, serine or glycine.

7. A Cutinase variant according to any of the preceding Claims, wherein the parent Cutinase is an eukaryotic Cutinase.

8. A Cutinase variant according to any one of the preceding Claims, in which the parent enzyme is a Cutinase which is immunologically cross-reacting with antibodies raised against the cutinase from Fusarium solani pisi.

9. A Cutinase variant according to any one of the preceding Claims, in which the parent enzyme is the cutinase from Fusarium solani pisi.

10. A Cutinase variant according to any one of the preceding Claims, whereby the modified residues are located in that part of the molecule which is defined by the vector which is the least-square fit through the Cα-atoms of residues 116 to 120 of the Fusarium solani pisi cutinase, or the corresponding Cα-atoms of a different Cutinase, and the plane perpendicular to said vector and containing the Cα-atom of residue 117, or the corresponding Cα-atom of a different Cutinase.

11. A Cutinase variant according to any one of the preceding Claims, in which the modified residues are located in that part of the molecule which is located between a first plane perpendicular to the vector which is the least-square fit through the Cα-atoms of residues 116 to 120 of the Fusarium solani pisi cutinase at a distance of 15 A from the Cα-atom of residue 117, and a second plane parallel to said first plane and containing the Cα-atom of residue 117.

12. A Cutinase variant according to any one of the preceding Claims, in which the modified residues are located at one or more of the following positions in the amino acid sequence of the Fusarium solani pisi cutinase, or the corresponding positions in a different Cutinase:
17, 18, 19, 40, 42-46, 50, 53, 54, 58, 74, 75, 76, 78, 80-88, 91, 92, 93, 95, 96, 97, 99, 100, 119, 150-156, 158, 160, 168, 169, 170, 172, 173, 174, 176, 179, 180-190, 192, 193, 194, 196, 197, 198, 201.

13. A Cutinase variant according to any one of the preceding Claims, in which the modified residues are located one or more of the following positions in the amino acid sequence of the Fusarium solani pisi cutinase, or the corresponding positions in a different Cutinase:
19, 41, 45, 49, 54, 58, 75, 76, 82, 85, 92, 93, 99, 100, 127, 128, 172, 173, 179, 183, 184, 185, 189, 190, 194, 197, 201.

14. A Cutinase variant according to any one of the preceding Claims, in which on or more of the following modifications have been effected in the amino acid sequence of the Fusarium solani pisi Cutinase, or the corresponding positions in a different Cutinase: T19V, G41A, T45K, T45P, *I49a, S54I, N58R, G75R, A76P, G82A, D83S, A85F, A85V, S92R, A93V, L99K, G100R, A127L, A128F, N172K, T173I, T179F, I183F, V184I, A185L, L189F, A190L, D194R, G197V, E201K.

15. A Cutinase variant according to any one of the preceding Claims, in which the following modification has been effected in the amino acid sequence of the Fusarium solani pisi Cutinase, or the corresponding positions in a different Cutinase: A85F, N172K, E201K.

16. A process for producing a Cutinase variant according to any one of the preceding Claims, which comprises the steps of fermentatively cultivating an rDNA modified microorganism containing a gene made by rDNA technique which encodes the Cutinase variant, making a preparation of the Cutinase variant by separating the Cutinase variant produced by the micro-organism either from the fermentation broth, or by separating the cells of the micro-organism from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the Cutinase either from said broth or from said cells by physical or chemical concentration or purification methods.

17. An rDNA modified micro-organism which has been transformed by a rDNA vector carrying a gene encoding a

Cutinase variant according to any of Claims 1 to 15 and which is thereby able to express said Cutinase variant.

18. An rDNA modified micro-organism according to Claim 17 carrying a gene encoding a Cutinase variant that is introduced into the micro-organism by fusion at its 5'-end to a gene fragment encoding a (modified) pre-sequence functional as a signal- or secretion-sequence for the host organism.

19. An rDNA modified micro-organism according to any one of Claims 17 or 18, wherein the host organism is a eukaryote, for example a yeast of the genus Saccharomyces or Kluyveromyces or the genus Hansenula, or a fungus of the genus Aspergillus.

20. An rDNA modified micro-organism according to any one of Claims 17 to 19, carrying a recombinant DNA vector coding for a Cutinase variant according to any of Claims 1 - 15, said micro-organism having being made an auxotrophic mutant by gene replacement of the gene coding for the auxotrophic marker in one of its ancestor cells.

21. A polynucleotide having a base sequence that encodes the mature Cutinase variant according to any one of Claims 1 - 15 or a functional equivalent or a mutant thereof, in which polynucleotide the final translated codon is followed by a stop codon and optionally having nucleotide sequences coding for the pre-sequence of this Cutinase directly upstream of the nucleotide sequences coding for the mature enzyme.

22. A polynucleotide having a base sequence encoding a Cutinase variant according to any of Claims 1 - 15, in which polynucleotide the final translated codon is followed by a stop codon and optionally having a nucleotide sequence coding for at least a part of the corresponding presequence, and/or a signal- or secretion-sequence suitable for a selected host organism, directly upstream of the nucleotide sequence coding for the mature enzyme.

23. A polynucleotide having a base sequence that encodes the mature Cutinase variant according to any one of Claims 1 - 15, or a functional equivalent or mutant thereof, in which the Cutinase-variant encoding nucleotide sequence derived from the organism of origin has been modified in such a way that at least one codon, and preferably as many codons as possible, have been made the subject of 'silent' mutations to form codons encoding equivalent amino acid residues and being codons preferred by a new host as specified in one of Claims 17 to 20, thereby to provide in use within the cells of such host a messenger-RNA for the introduced gene of improved stability.

24. A polynucleotide according to any one of Claims 21 to 23, in which upstream of the nucleotide sequences coding for the pro-or mature Cutinase variant, there is located a nucleotide sequence that codes for a signal or secretion sequence suitable for a host as specified in any one of Claims 17 to 20.

25. A recombinant DNA vector able to direct the expression of a nucleotide sequence encoding a Cutinase variant gene, comprising the following components:
(a) Double-stranded (ds) DNA coding for the mature Cutinase variant or precutinase or a corresponding precutinase in which at least part of the presequence has been removed directly down stream of a secretion signal (preferred for the selected host cell), provided that where the part of the gene that should be translated does not start with the codon ATG, an ATG codon should be placed in front, and provided also that the part of the gene to be translated ends with an appropriate stop codon or has such codon added;
(b) An expression regulon (suitable for the selected host organism) situated upstream of the plus strand of the ds DNA encoding the Cutinase variant (component (a));
(c) A terminator sequence (suitable for the selected host organism) situated down stream of the plus strand of the ds DNA encoding the Cutinase variant (component (a);

(d1) Nucleotide sequences which facilitate integration, of the ds DNA into the genome of the selected host or,
(d2) an origin of replication suitable for the selected host;
(e1) Optionally a (auxotrophic) selection marker;
(e2) Optionally a ds DNA sequence encoding proteins involved in the maturation and/or secretion of one of the precursor forms of the Cutinase variant in the host selected.

26. A recombinant DNA vector according to Claim 25, also carrying, upstream and/or downstream of the polynucleotide as earlier defined, further sequences facilitative of functional expression of the Cutinase.

27. A recombinant DNA vector according to any one of Claims 25 - 26, carrying an auxotrophic marker consisting of a coding region of the auxotrophic marker and a defective promotor region.

28. An enzymatic detergent composition comprising a Cutinase variant according to any one of Claims 1 to 15.

*****

Fig.1A.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE I

CODE	Length	5' <--- sequence ---> 3'
CUTI1A IG	(44)	aat tcg agc tca tcA TGA AAT TCT TCG CGT TAA CCA CAC TTC TC
CUTI1B IG	(39)	GCC GCC ACG GCT TCG GCT CTG CCT ACT AGT AAC CCT GCT
CUTI1C IG	(42)	CAG GAG CTT GAG GCG CGC CAG CTT GGT AGA ACA ACT CGC GAC
CUTI1D IG	(39)	GAT CTG ATC AAC GGC AAT AGC GCT TCC TGC GCC GAT GTC
CUTI1E IG	(33)	ATC TTC ATT TAT GCT CGA GGT TCA ACA GAG ACG
CUTI1F IG	(28)	GGC AAC TTG GGA ACT CTC GGG CCC AGC A
CUTI1G IG	(31)	GGT TAA CGC GAA GAA TTT CAT gat gag ctc g
CUTI1H IG	(39)	ACT AGT AGG CAG AGC CGA AGC CGT GGC GGC GAG AAG TGT
CUTI1I IG	(42)	TGT TCT ACC AAG CTG GCG CGC CTC AAG CTC CTG AGC AGG GTT
CUTI1J IG	(39)	GCA GGA AGC GCT ATT GCC GTT GAT CAG ATC GTC GCG AGT
CUTI1K IG	(33)	TGA ACC TCG AGC ATA AAT GAA GAT GAC ATC GGC
CUTI1L IG	(41)	AGC TTG CTG GGC CCG AGA GTT CCC AAG TTG CCC GTC TCT GT

```
        10          20          30          40          50          60
               aattcg agctcatcAT GAAATTCTTC GCGTTAACCA CACTTCTCGC
                   gc tcgagtagTA CTTTAAGAAG CGCAATTGGT GTGAAGAGCG

        70          80          90         100         110         120
CGCCACGGCT TCGGCTCTGC CTACTAGTAA CCCTGCTCAG GAGCTTGAGG CGCGCCAGCT
GCGGTGCCGA AGCCGAGACG GATGATCATT GGGACGAGTC CTCGAACTCC GCGCGGTCGA

       130         140         150         160         170         180
TGGTAGAACA ACTCGCGACG ATCTGATCAA CGGCAATAGC GCTTCCTGCG CCGATGTCAT
ACCATCTTGT TGAGCGCTGC TAGACTAGTT GCCGTTATCG CGAAGGACGC GGCTACAGTA

       190         200         210         220         230         240
CTTCATTTAT GCTCGAGGTT CAACAGAGAC GGGCAACTTG GGAACTCTCG GGCCCAGCA
GAAGTAAATA CGAGCTCCAA GTTGTCTCTG CCCGTTGAAC CCTTGAGAGC CCGGGTCGTT

       250

GGA
```

Fig.1B.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE 2

CODE	Length	5' <--- sequence ---> 3'
CUTI2A MH	(40)	AAT TCT CGG GCC CAG CAT TGC CTC CAA CCT TGA GTC CGC C
CUTI2B MH	(36)	TTC GGC AAG GAC GGT GTC TGG ATT CAG GGC GTT GGC
CUTI2C MH	(36)	GGT GCC TAC CGA GCC ACC CTA GGA GAC AAT GCT CTC
CUTI2D MH	(39)	CCG CGG GGA ACC TCT AGC GCC GCA ATC AGG GAG ATG CTA
CUTI2E MH	(45)	GGC CTC TTC CAG CAG GCC AAC ACC AAG TGC CCT GAC GCG ACT TTG
CUTI2F MH	(46)	ATC GCC GGT GGC TAC AGC CAG GGT GCT GCA CTT GCA GCC GCT AGC A
CUTI2G MH	(45)	CTT GCC GAA GGC GGA CTC AAG GTT GGA GGC AAT GCT GGG CCC GAG
CUTI2H MH	(36)	GTA GGC ACC GCC AAC GCC CTG AAT CCA GAC ACC GTC
CUTI2I MH	(36)	TCC CCG CGG GAG AGC ATT GTC TCC TAG GGT GGC TCG
CUTI2J MH	(39)	GAA GAG GCC TAG CAT CTC CCT GAT TGC GGC GCT AGA GGT
CUTI2K MH	(45)	ACC GGC GAT CAA AGT CGC GTC AGG GCA CTT GGT GTT GGC CTG CTG
CUTI2L MH	(41)	AGC TTG CTA GCG GCT GCA AGT GCA GCA CCC TGG CTG TAG CC

```
      10         20         30         40         50         60
  AATTCTC GGGCCCAGCA TTGCCTCCAA CCTTGAGTCC GCCTTCGGCA AGGACGGTGT
      GAG CCCGGGTCGT AACGGAGGTT GGAACTCAGG CGGAAGCCGT TCCTGCCACA

      70         80         90        100        110        120
CTGGATTCAG GGCGTTGGGG GTGCCTACCG AGCCACCCTA GGAGACAATG CTCTCCCGCG
GACCTAAGTC CCGCAACCCC CACGGATGGC TCGGTGGGAT CCTCTGTTAC GAGAGGGCGC

     130        140        150        160        170        180
GGGAACCTCT AGCGCCGCAA TCAGGGAGAT GCTAGGCCTC TTCCAGCAGG CCAACACCAA
CCCTTGGAGA TCGCGGCGTT AGTCCCTCTA CGATCCGGAG AAGGTCGTCC GGTTGTGGTT

     190        200        210        220        230        240
GTGCCCTGAC GCGACTTTGA TCGCCGGTGG CTACAGCCAG GGTGCTGCAC TTGCAGCCGC
CACGGGACTG CGCTGAAACT AGCGGCCACC GATGTCGGTC CCACGACGTG AACGTCGGCG

      250
TAGCA
ATCGTTCGA
```

Fig.1C.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE 3

CODE	Length	5' <--- sequence ---> 3'
CUTI3A	MH (43)	AAT TCC CGC TAG CAT CGA GGA CCT CGA CTC GGC CAT TCG TGA C
CUTI3B	MH (45)	AAG ATC GCC GGT ACC GTT CTG TTC GGC TAC ACC AAG AAC CTA CAG
CUTI3C1	MH (42)	AAT CGC GGC CGA ATC CCC AAC TAC CCT GCC GAC AGG ACC AAG
CUTI3D	MH (42)	GTC TTC TGC AAT ACA GGA GAT CTC GTT TGT ACT GGT AGC TTG
CUTI3E	MH (39)	ATC GTT GCT GCA CCT CAC TTG GCA TAT GGT CCT GAT GCC
CUTI3F	MH (33)	CGG GGA CCT GCC CCT GAG TTC CTC ATC GAG AAG
CUTI3G1	MH (32)	GTT CGG GCT GTC CGT GGT TCT GCT TGA gct ta
CUTI3H	MH (30)	GGC CGA GTC GAG GTC CTC GAT GCT AGC GGG
CUTI3I	MH (45)	CTT GGT GTA GCC GAA CAG AAC GGT ACC GGC GAT CTT GTC ACG AAT
CUTI3J1	MH (42)	GTC GGC AGG GTA GTT GGG GAT TCG GCC GCG ATT CTG TAG GTT
CUTI3K	MH (42)	AGT ACA AAC GAG ATC TCC TGT ATT GCA GAA GAC CTT GGT CCT
CUTI3L	MH (39)	ACC ATA TGC CAA GTG AGG TGC AGC AAC GAT CAA GCT ACC
CUTI3M	MH (33)	GAG GAA CTC AGG GGC AGG TCC CCG GGC ATC AGG
CUTI3N1	MH (45)	agc tta agc TCA AGC AGA ACC ACG GAC AGC CCG AAC CTT CTC GAT

```
        10          20          30          40          50          60
 AATTCCCGC TAGCATCGAG GACCTCGACT CGGCCATTCG TGACAAGATC GCCGGTACCG
    GGGCG ATCGTAGCTC CTGGAGCTGA GCCGGTAAGC ACTGTTCTAG CGGCCATGGC

        70          80          90          100         110         120
TTCTGTTCGG CTACACCAAG AACCTACAGA ATCGCGGCCG AATCCCCAAC TACCCTGCCG
AAGACAAGCC GATGTGGTTC TTGGATGTCT TAGCGCCGGC TTAGGGGTTG ATGGGACGGC

        130         140         150         160         170         180
ACAGGACCAA GGTCTTCTGC AATACAGGAG ATCTCGTTTG TACTGGTAGC TTGATCGTTG
TGTCCTGGTT CCAGAAGACG TTATGTCCTC TAGAGCAAAC ATGACCATCG AACTAGCAAC

        190         200         210         220         230         240
CTGCACCTCA CTTGGCATAT GGTCCTGATG CCCGGGGACC TGCCCCTGAG TTCCTCATCG
GACGTGGAGT GAACCGTATA CCAGGACTAC GGGCCCCTGG ACGGGGACTC AAGGAGTAGC

        250         260         270         280
AGAAGGTTCG GGCTGTCCGT GGTTCTGCTT GAgctta
TCTTCCAAGC CCGACAGGCA CCAAGACGAA CTcgaattcg a
```

Fig.1D(1 of 4).

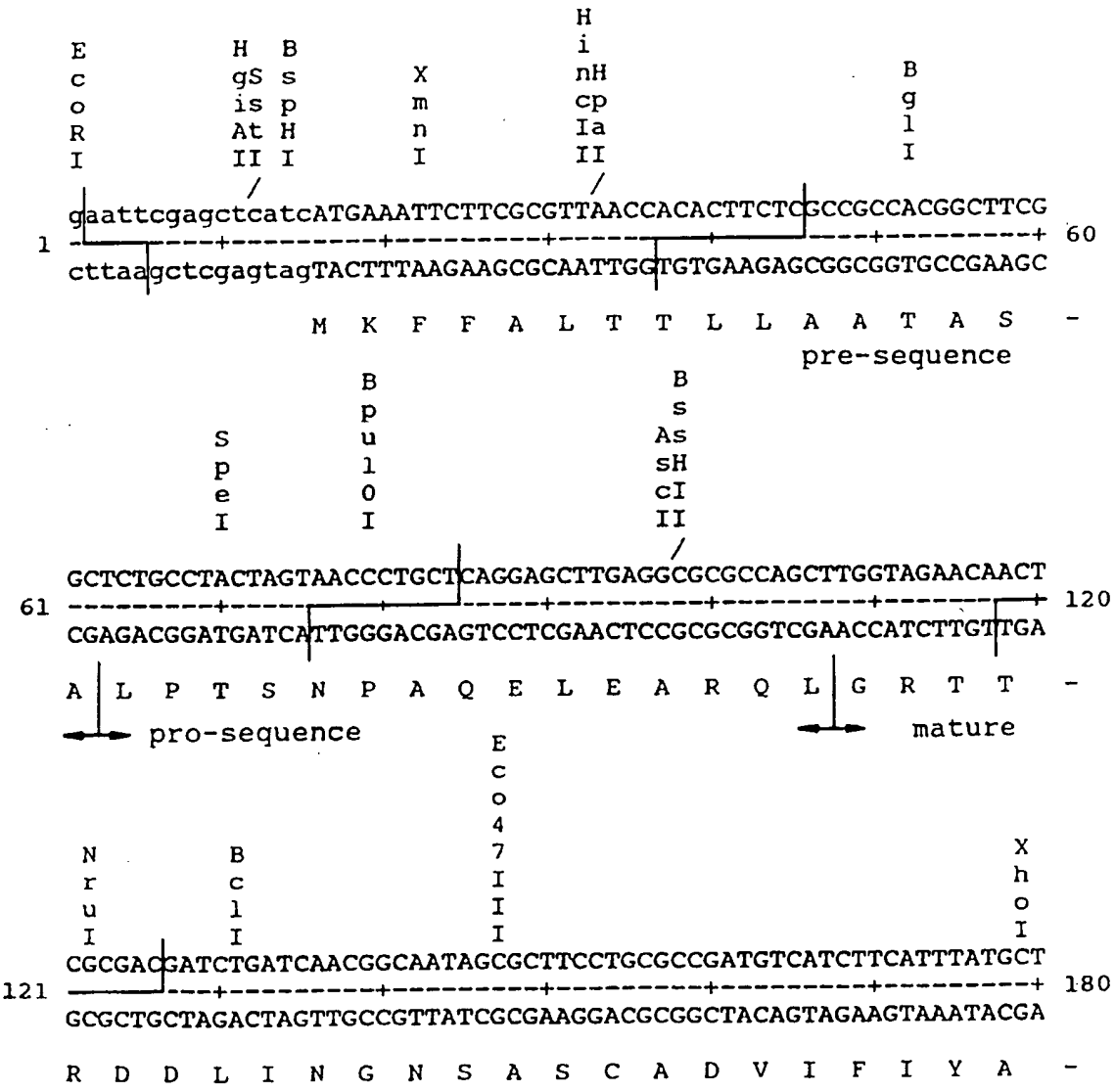

EcoRI
HgsIsAtII
BsspHI
XmnI
HinHcpIaII
BglI
gaattcgagctcatcATGAAATTCTTCGCGTTAACCACACTTCTCGCCGCCACGGCTTCG
cttaagctcgagtagTACTTTAAGAAGCGCAATTGGTGTGAAGAGCGGCGGTGCCGAAGC
1
60
M K F F A L T T L L A A T A S -
pre-sequence
SpeI
BpuI0I
BssAsHcII
GCTCTGCCTACTAGTAACCCTGCTCAGGAGCTTGAGGCGCGCCAGCTTGGTAGAACAACT
CGAGACGGATGATCATTGGGACGAGTCCTCGAACTCCGCGCGGTCGAACCATCTTGTTGA
61
120
A L P T S N P A Q E L E A R Q L G R T T -
pro-sequence
mature
NruI
BclI
Eco47III
XhoI
CGCGACGATCTGATCAACGGCAATAGCGCTTCCTGCGCCGATGTCATCTTCATTTATGCT
GCGCTGCTAGACTAGTTGCCGTTATCGCGAAGGACGCGGCTACAGTAGAAGTAAATACGA
121
180
R D D L I N G N S A S C A D V I F I Y A -

Fig.1D(2 of 4)

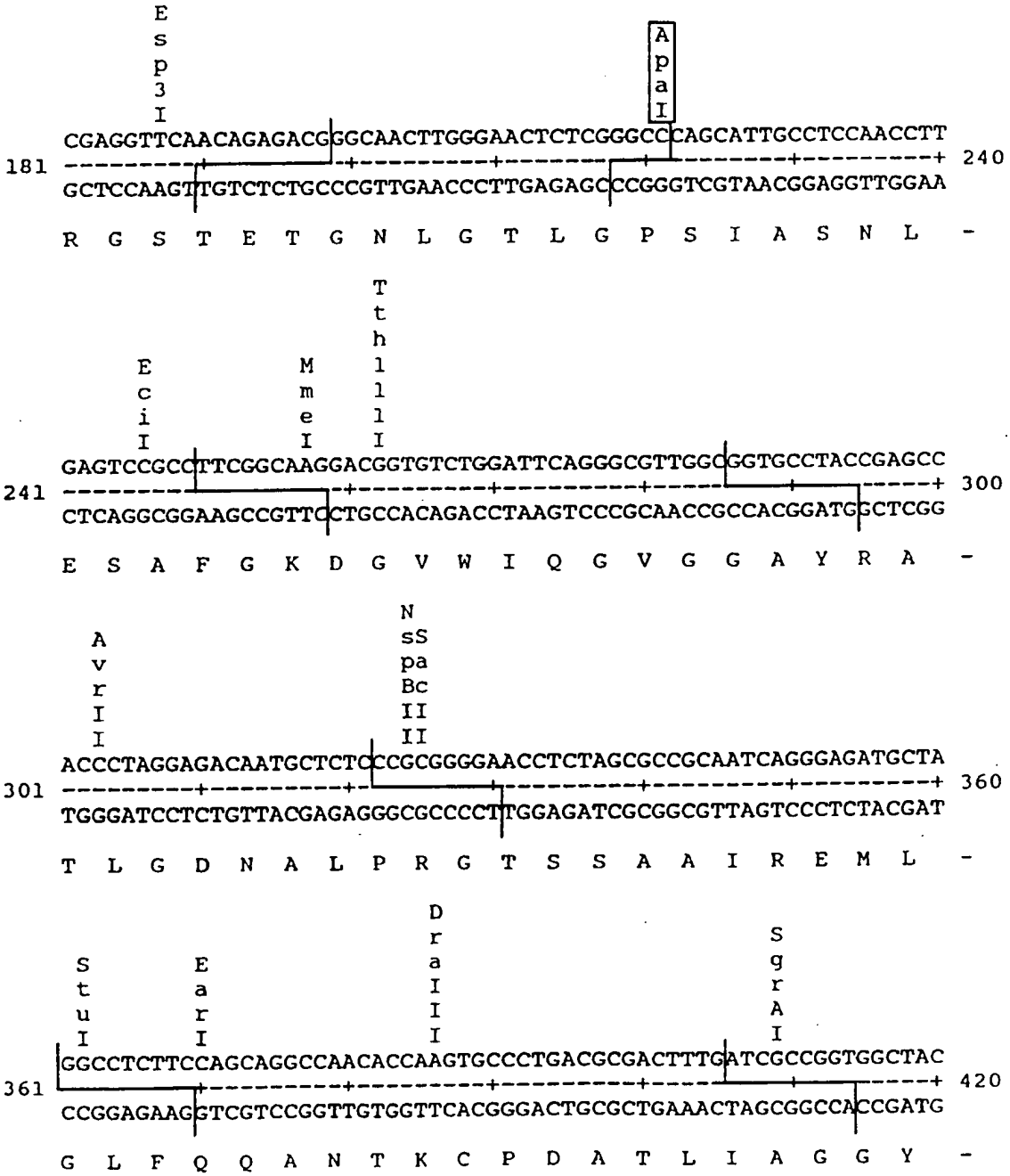

Esp3I
ApaI
CGAGGTTCAACAGAGACGGGCAACTTGGGAACTCTCGGGCCCAGCATTGCCTCCAACCTT
181
GCTCCAAGTTGTCTCTGCCCGTTGAACCCTTGAGAGCCCGGGTCGTAACGGAGGTTGGAA 240
R G S T E T G N L G T L G P S I A S N L -
ThhIII
EciI
MmeI
GAGTCCGCCTTCGGCAAGGACGGTGTCTGGATTCAGGGCGTTGGCGGTGCCTACCGAGCC
241
CTCAGGCGGAAGCCGTTCCTGCCACAGACCTAAGTCCCGCAACCGCCACGGATGGCTCGG 300
E S A F G K D G V W I Q G V G G A Y R A -
NsSpaBcII
AvrII
ACCCTAGGAGACAATGCTCTCCCGCGGGGAACCTCTAGCGCCGCAATCAGGGAGATGCTA
301
TGGGATCCTCTGTTACGAGAGGGCGCCCCTTGGAGATCGCGGCGTTAGTCCCTCTACGAT 360
T L G D N A L P R G T S S A A I R E M L -
DraIII
SgrAI
StuI
EarI
GGCCTCTTCCAGCAGGCCAACACCAAGTGCCCTGACGCGACTTTGATCGCCGGTGGCTAC
361
CCGGAGAAGGTCGTCCGGTTGTGGTTCACGGGACTGCGCTGAAACTAGCGGCCACCGATG 420
G L F Q Q A N T K C P D A T L I A G G Y -

Fig.1D(3 of 4)

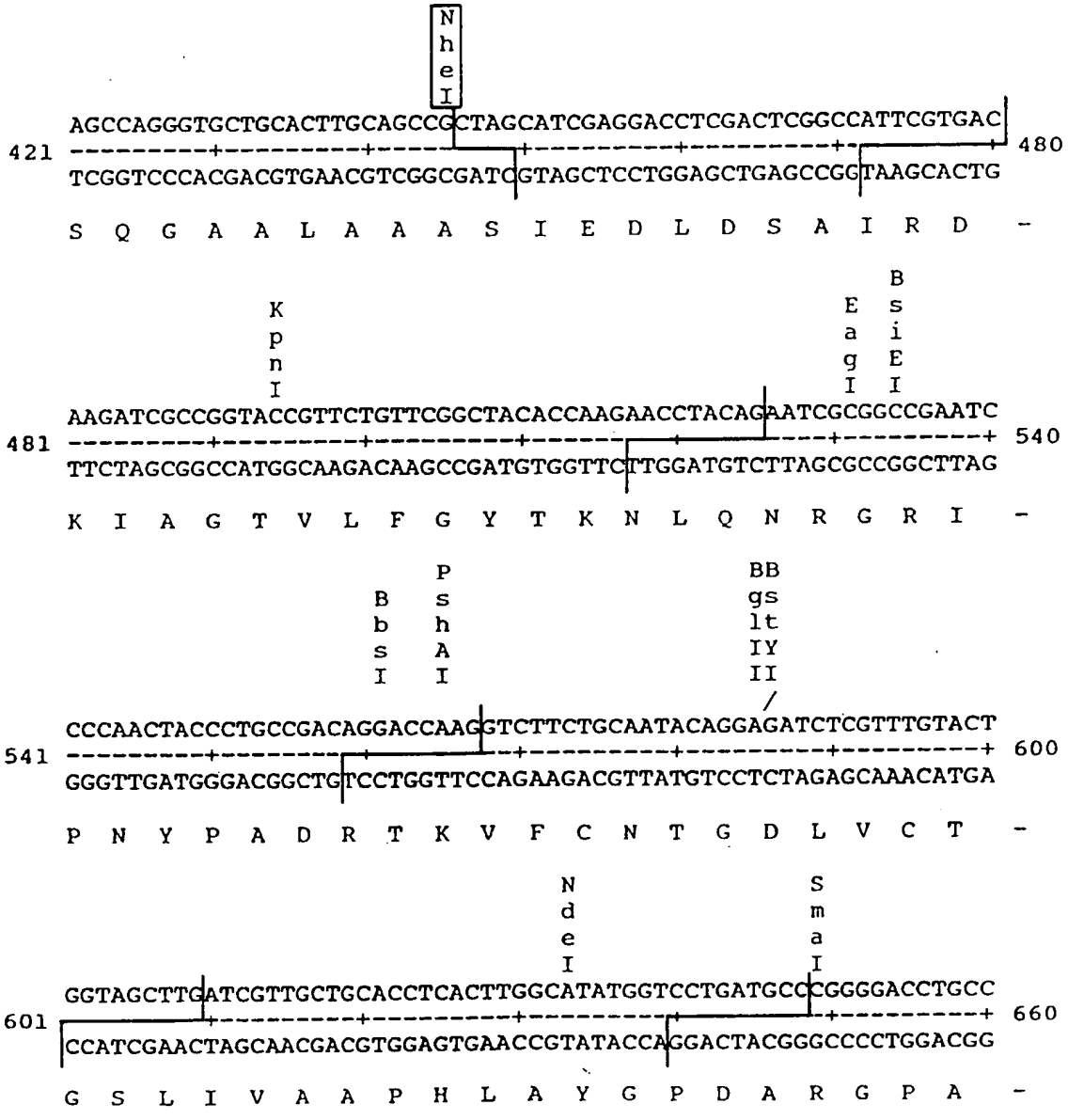

NheI
AGCCAGGGTGCTGCACTTGCAGCCGCTAGCATCGAGGACCTCGACTCGGCCATTCGTGAC
421
480
TCGGTCCCACGACGTGAACGTCGGCGATCGTAGCTCCTGGAGCTGAGCCGGTAAGCACTG
S Q G A A L A A A S I E D L D S A I R D -
KpnI
EagI
BsiEI
AAGATCGCCGGTACCGTTCTGTTCGGCTACACCAAGAACCTACAGAATCGCGGCCGAATC
481
540
TTCTAGCGGCCATGGCAAGACAAGCCGATGTGGTTCTTGGATGTCTTAGCGCCGGCTTAG
K I A G T V L F G Y T K N L Q N R G R I -
BbsI
PshAI
BBgsltIYII
CCCAACTACCCTGCCGACAGGACCAAGGTCTTCTGCAATACAGGAGATCTCGTTTGTACT
541
600
GGGTTGATGGGACGGCTGTCCTGGTTCCAGAAGACGTTATGTCCTCTAGAGCAAACATGA
P N Y P A D R T K V F C N T G D L V C T -
NdeI
SmaI
GGTAGCTTGATCGTTGCTGCACCTCACTTGGCATATGGTCCTGATGCCCGGGGACCTGCC
601
660
CCATCGAACTAGCAACGACGTGGAGTGAACCGTATACCAGGACTACGGGCCCCTGGACGG
G S L I V A A P H L A Y G P D A R G P A -

Fig.1D(4 of 4)

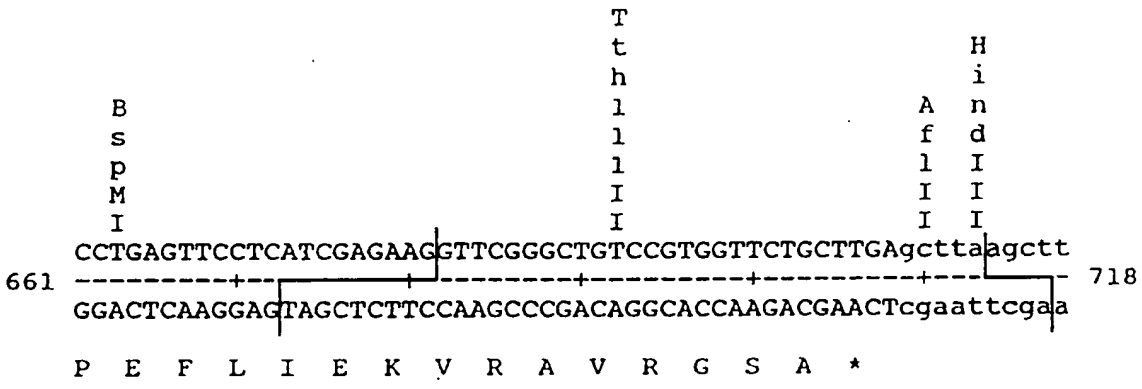

T
t
h
B
l
A
H
s
l
f
i
p
l
d
M
I
I
I
I
I
I
CCTGAGTTCCTCATCGAGAAGGTTCGGGCTGTCCGTGGTTCTGCTTGAgcttaagctt
661
718
GGACTCAAGGAGTAGCTCTTCCAAGCCCGACAGGCACCAAGACGAACTcgaattcgaa
P E F L I E K V R A V R G S A *

Fig. 2.

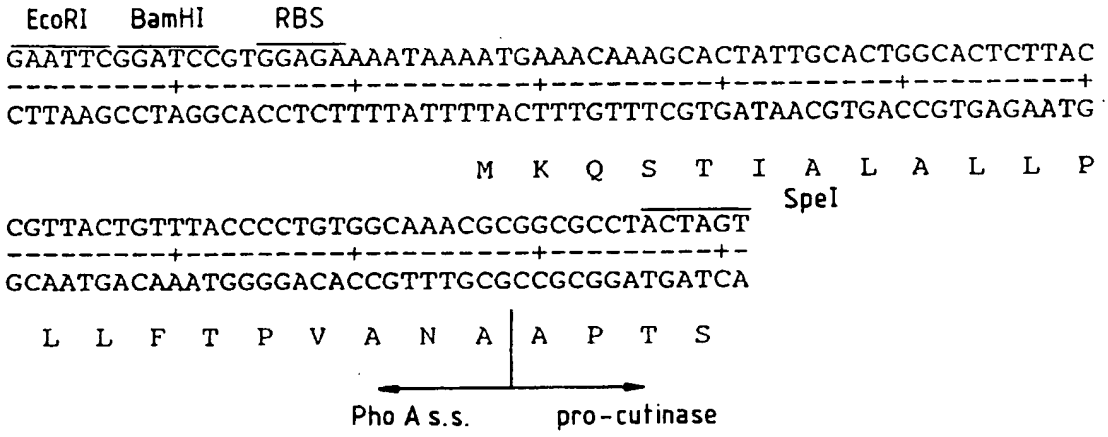

EcoRI
BamHI
RBS
GAATTCGGATCCGTGGAGAAAATAAAATGAAACAAAGCACTATTGCACTGGCACTCTTAC
CTTAAGCCTAGGCACCTCTTTTATTTTACTTTGTTTCGTGATAACGTGACCGTGAGAATG
M K Q S T I A L A L L P
SpeI
CGTTACTGTTTACCCCTGTGGCAAACGCGGCGCCTACTAGT
GCAATGACAAATGGGGACACCGTTTGCGCCGCGGATGATCA
L L F T P V A N A A P T S
Pho A s.s.
pro-cutinase

Fig. 3.

SYNTHETIC OLIGONULEOTIDES USED TO CONSTRUCT CASSETTE 8

CODE	Length	5' <--- sequence ---> 3'
		SacI
AC 01 cv	(38)	AAT TCT CGA GCT CAT CAC ACA AAC AAA CAA
		AAC AAA AT
AC 02 CV	(25)	GAT GCT TTT GCA AGC CTT CCT TTT C
AC 03 CV	(39)	CTT TTG GCT GGT TTT GCA GCC AAA ATA TCT
		GCG GGT AGA BclI
AC 04 CV	(25)	ACA ACT CGC GAC GAT CTG ATC ATC A
AC 05 CV	(41)	AGC TTG ATG ATC AGA TCG TCG CGA GTT GTT
		CTA CCC GCA GA
AC 06 CV	(17)	TAT TTT GGC TGC AAA AC
AC 07 CV	(46)	CAG CCA AAA GGA AAA GGA AGG CTT GCA AAA
		GCA TCA TTT TGT TTT G
AC 08 CV	(23)	TTT GTT TGT GTG ATG AGC TCG AG

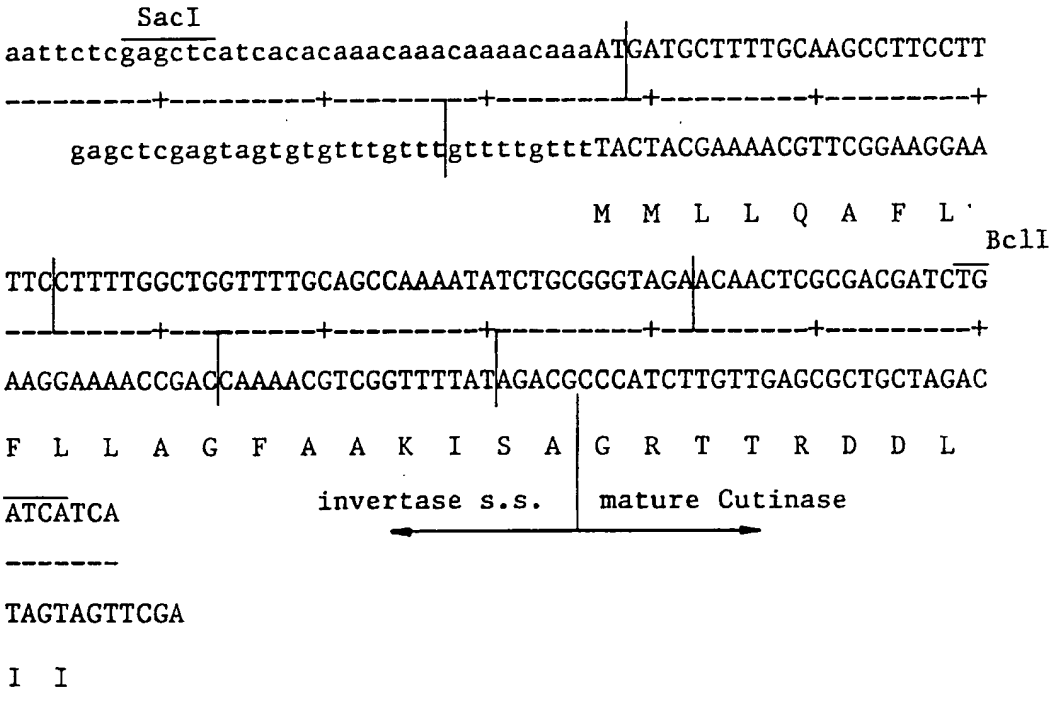


Fig.4.

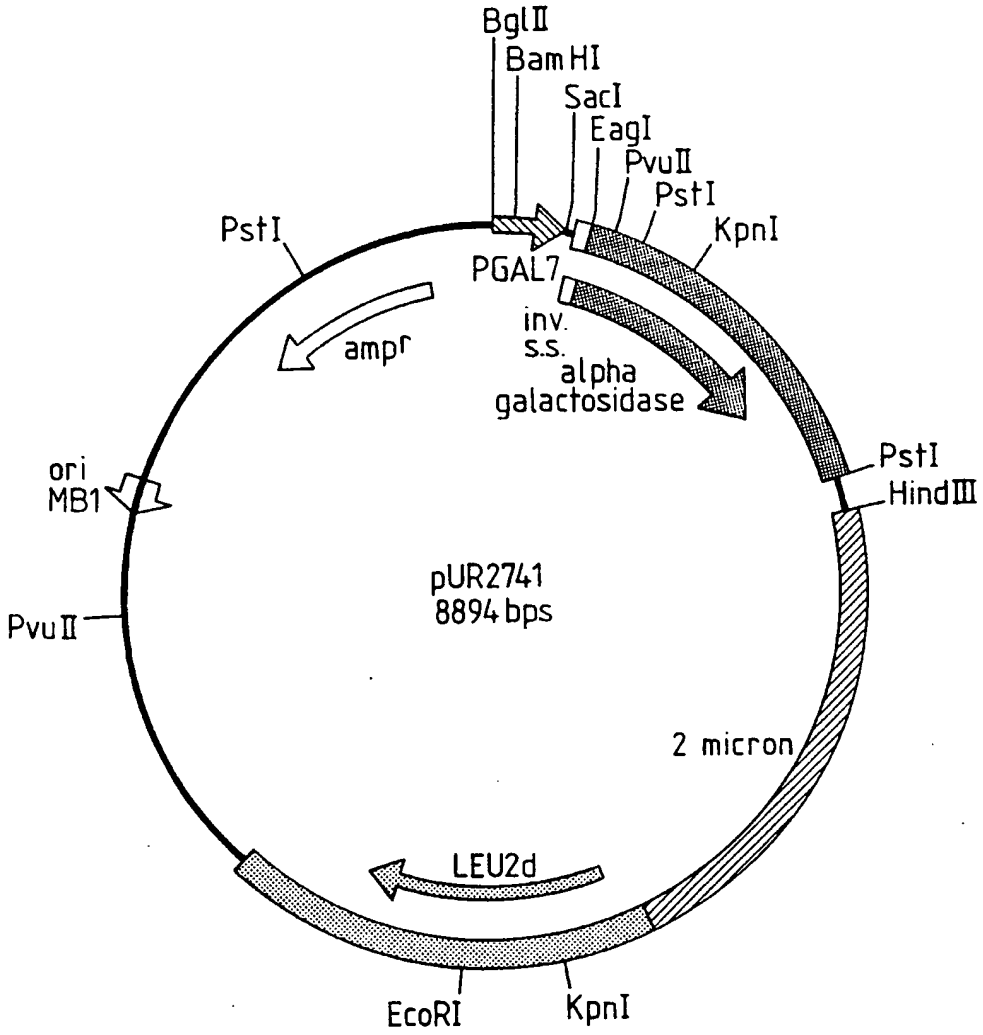

BglII
BamHI
SacI
EagI
PvuII
PstI
KpnI
PstI
PGAL7
inv.
s.s.
alpha
galactosidase
ampr
ori
MB1
PstI
HindIII
pUR2741
8894 bps
PvuII
2 micron
LEU2d
EcoRI
KpnI

Fig. 5.

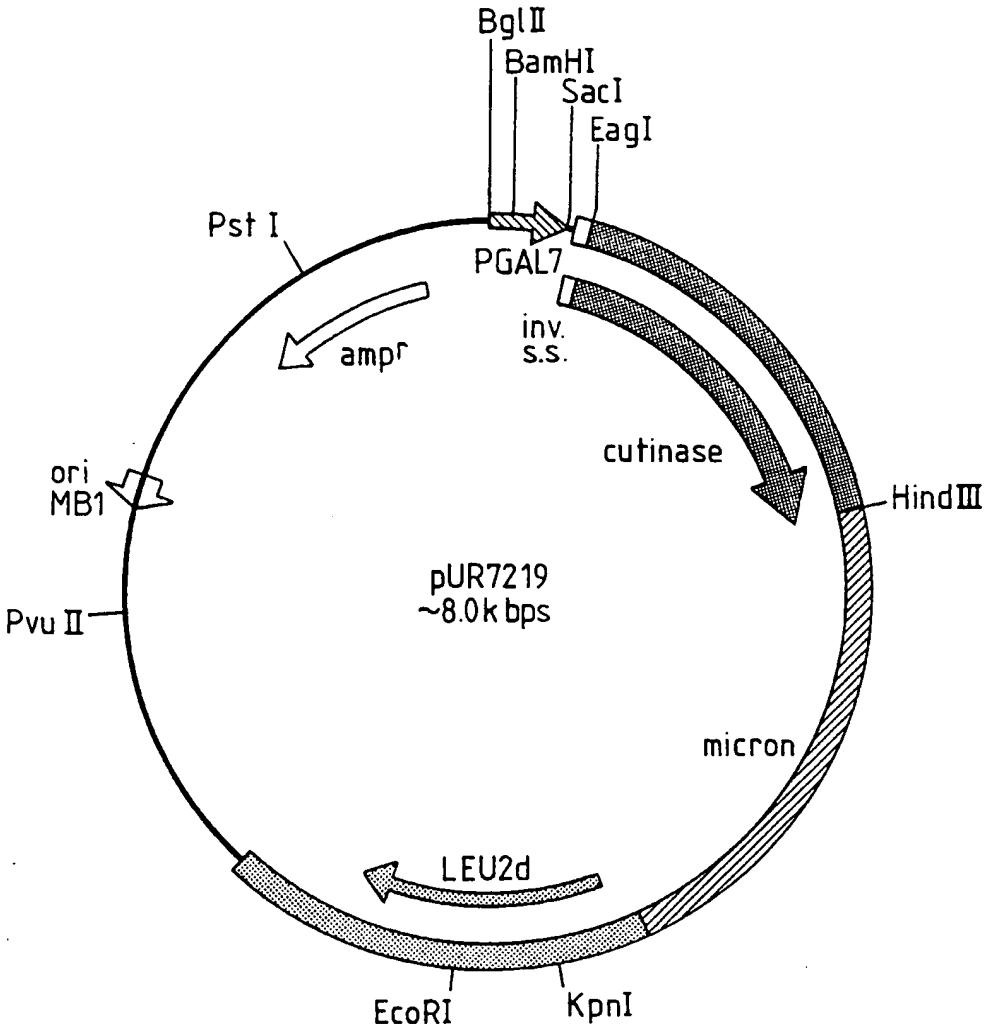

BglII
BamHI
SacI
EagI
Pst I
PGAL7
inv.
s.s.
ampr
cutinase
HindIII
ori
MB1
pUR7219
~8.0k bps
Pvu II
micron
LEU2d
EcoRI
KpnI

Fig. 6.

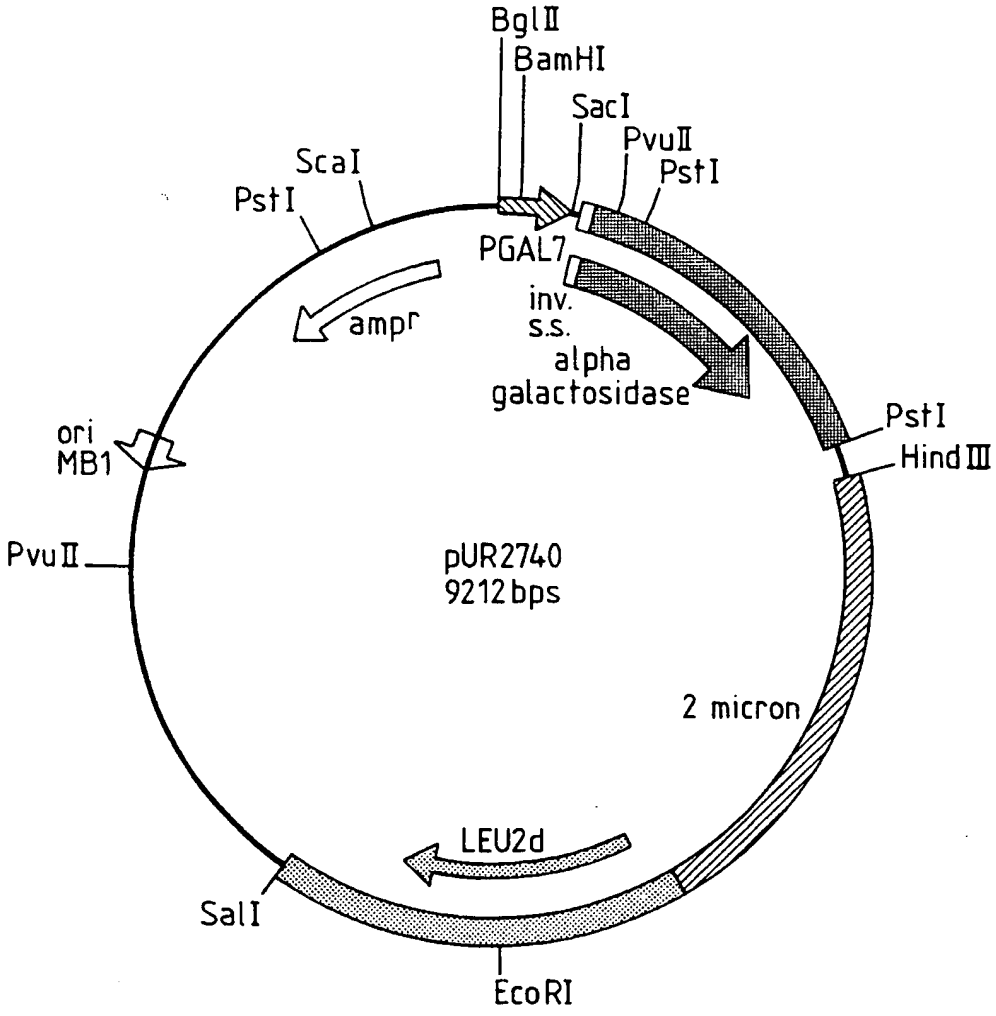

BglII
BamHI
SacI
PvuII
PstI
ScaI
PstI
PGAL7
inv.
s.s.
alpha
galactosidase
ampr
ori
MB1
PvuII
pUR2740
9212bps
PstI
HindIII
2 micron
LEU2d
SalI
EcoRI

Fig. 7.

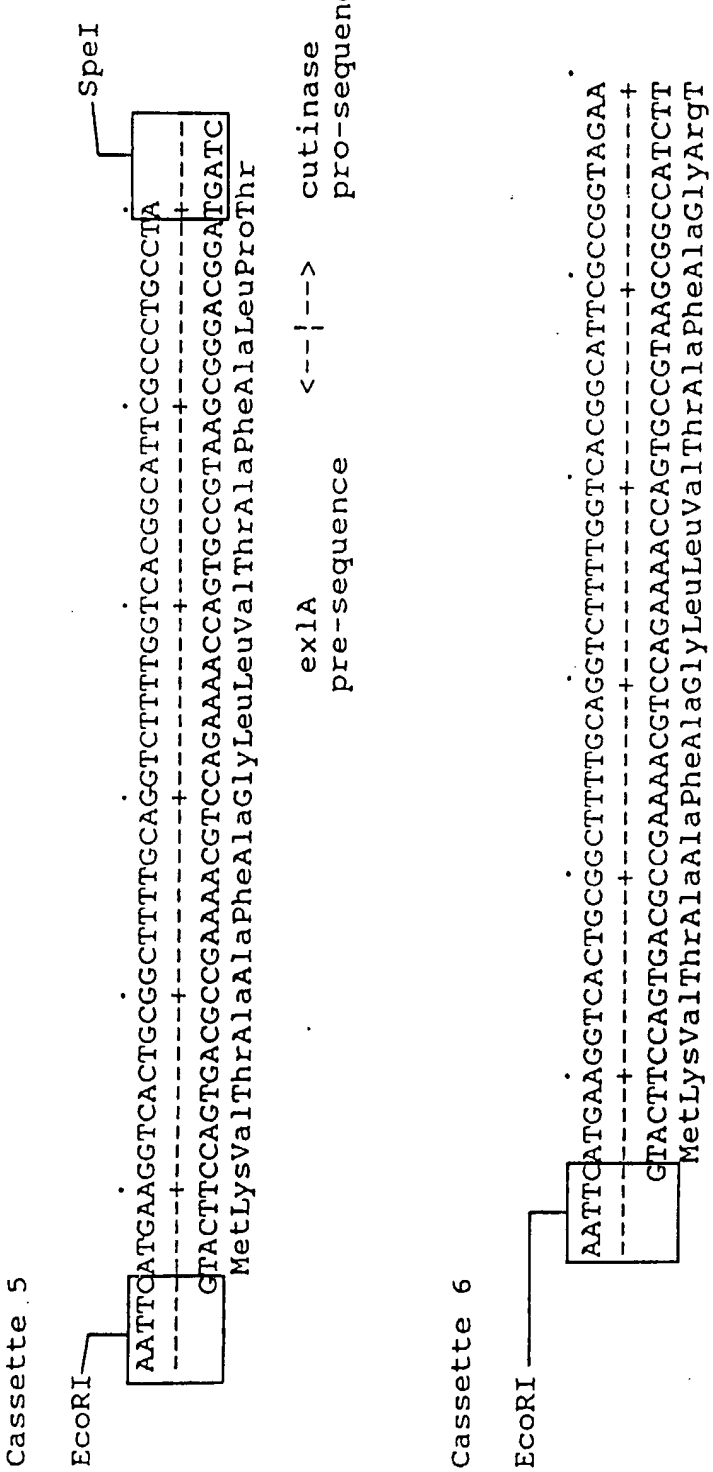

Cassette 5
EcoRI
SpeI
AATTCATGAAGGTCACTGCGGCTTTTGCAGGTCTTTTGGTCACGGCATTCGCCCTGCCCTA
GTACTTCCAGTGACGCCGAAAACGTCCAGAAAACCAGTGCCGTAAGCGGGACGGGATGATC
MetLysValThrAlaAlaPheAlaGlyLeuLeuValThrAlaPheAlaLeuProThr
exlA
pre-sequence
<--¦-->
cutinase
pro-sequence
Cassette 6
EcoRI
AATTCATGAAGGTCACTGCGGCTTTTGCAGGTCTTTTGGTCACGGCATTCGCCGGTAGAA
GTACTTCCAGTGACGCCGAAAACGTCCAGAAAACCAGTGCCGTAAGCGGCCATCTT
MetLysValThrAlaAlaPheAlaGlyLeuLeuValThrAlaPheAlaGlyArgT
exlA
pre-sequence
<--¦-->
cutinase
pro-sequence

Fig. 7(Cont).

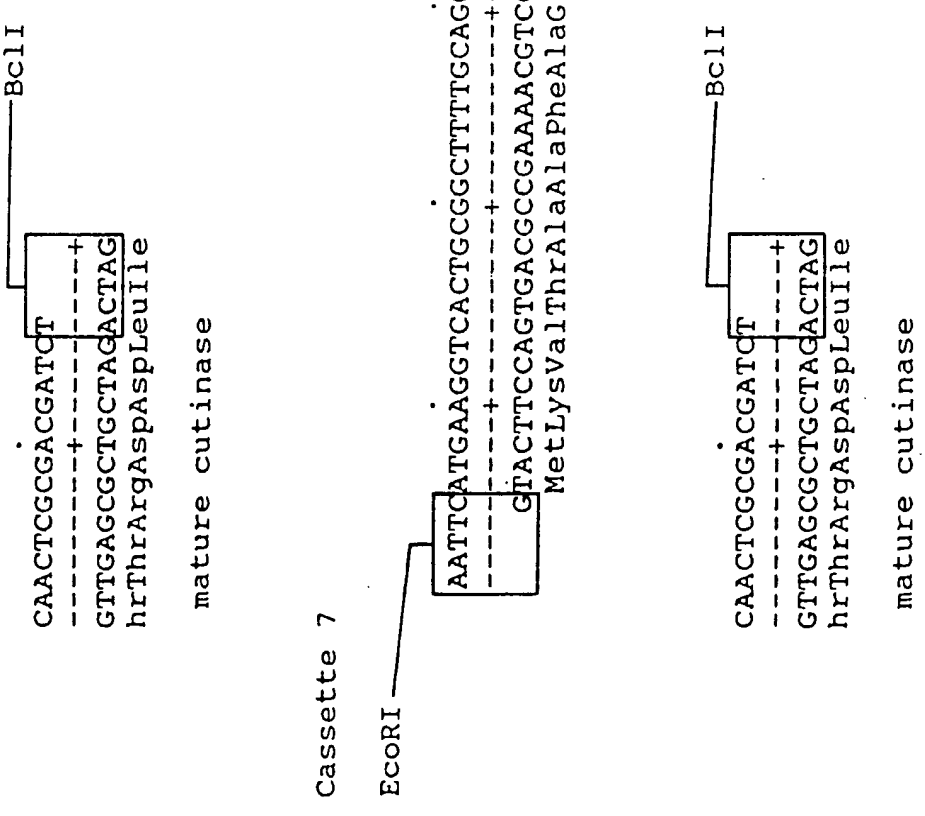

BclI
CAACTCGCGACGATCT
GTTGAGCGCTGCTAGACTAG
hrThrArgAspAspLeuIle
mature cutinase
Cassette 7
EcoRI
AATTCATGAAGGTCACTGCGGCTTTTGCAGGTCTTTTGGTCACGGCATTCGCCTCAAGAA
GTACTTCCAGTGACGCCGAAAACGTCCAGAAAACCAGTGCCGTAAGCGGAGTTCTT
MetLysValThrAlaAlaPheAlaGlyLeuLeuValThrAlaPheAlaSerArgT
exlA
pre-sequence
<--
-->
BclI
CAACTCGCGACGATCT
GTTGAGCGCTGCTAGACTAG
hrThrArgAspAspLeuIle
mature cutinase

Fig. 8.

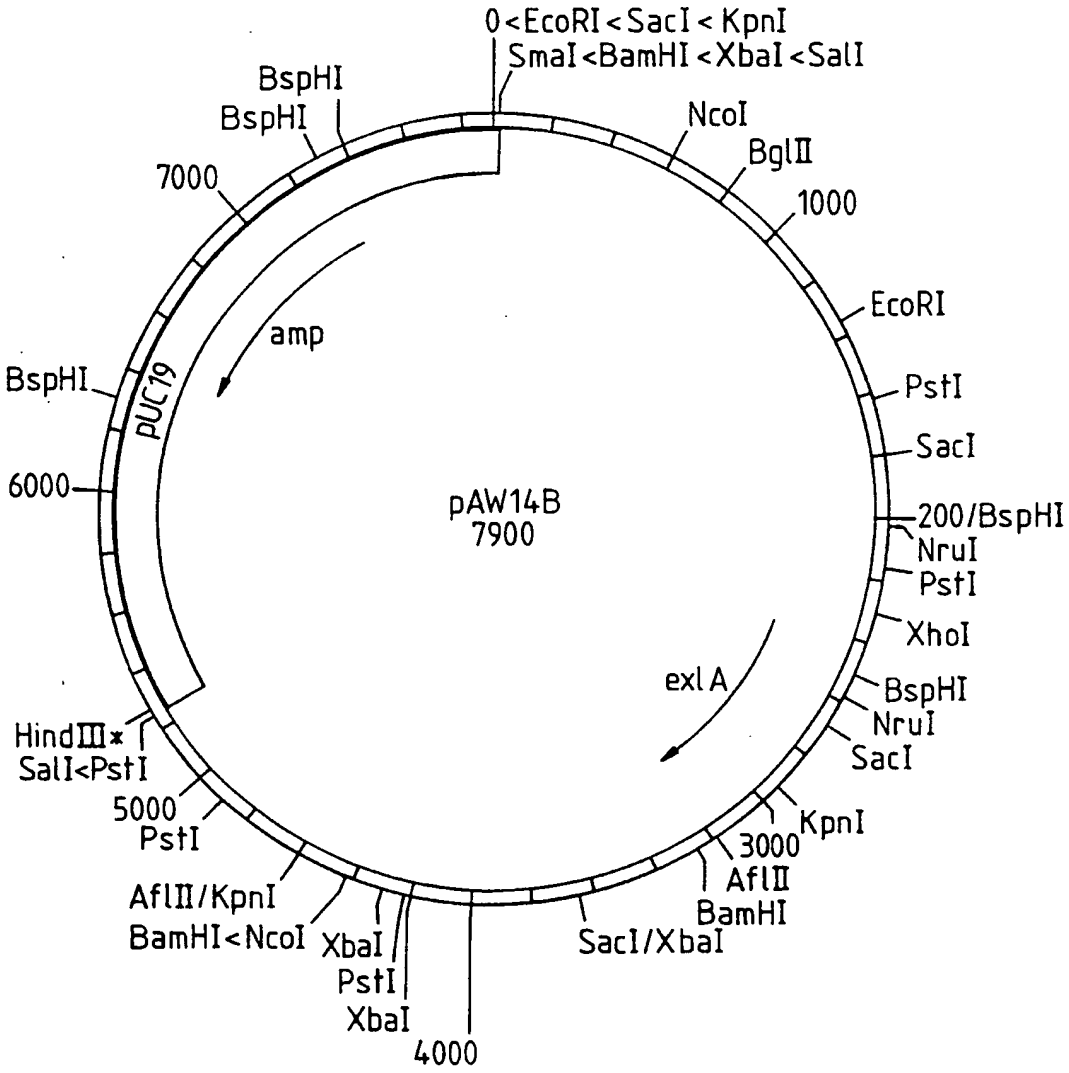

0<EcoRI<SacI<KpnI
SmaI<BamHI<XbaI<SalI
BspHI
BspHI
NcoI
BglII
1000
7000
EcoRI
amp
PstI
BspHI
pUC19
SacI
6000
pAW14B
7900
200/BspHI
NruI
PstI
XhoI
exl A
BspHI
NruI
SacI
HindIII*
SalI<PstI
KpnI
5000
PstI
3000
AflII
BamHI
AflII/KpnI
BamHI<NcoI
XbaI
SacI/XbaI
PstI
XbaI
4000

Fig. 9.

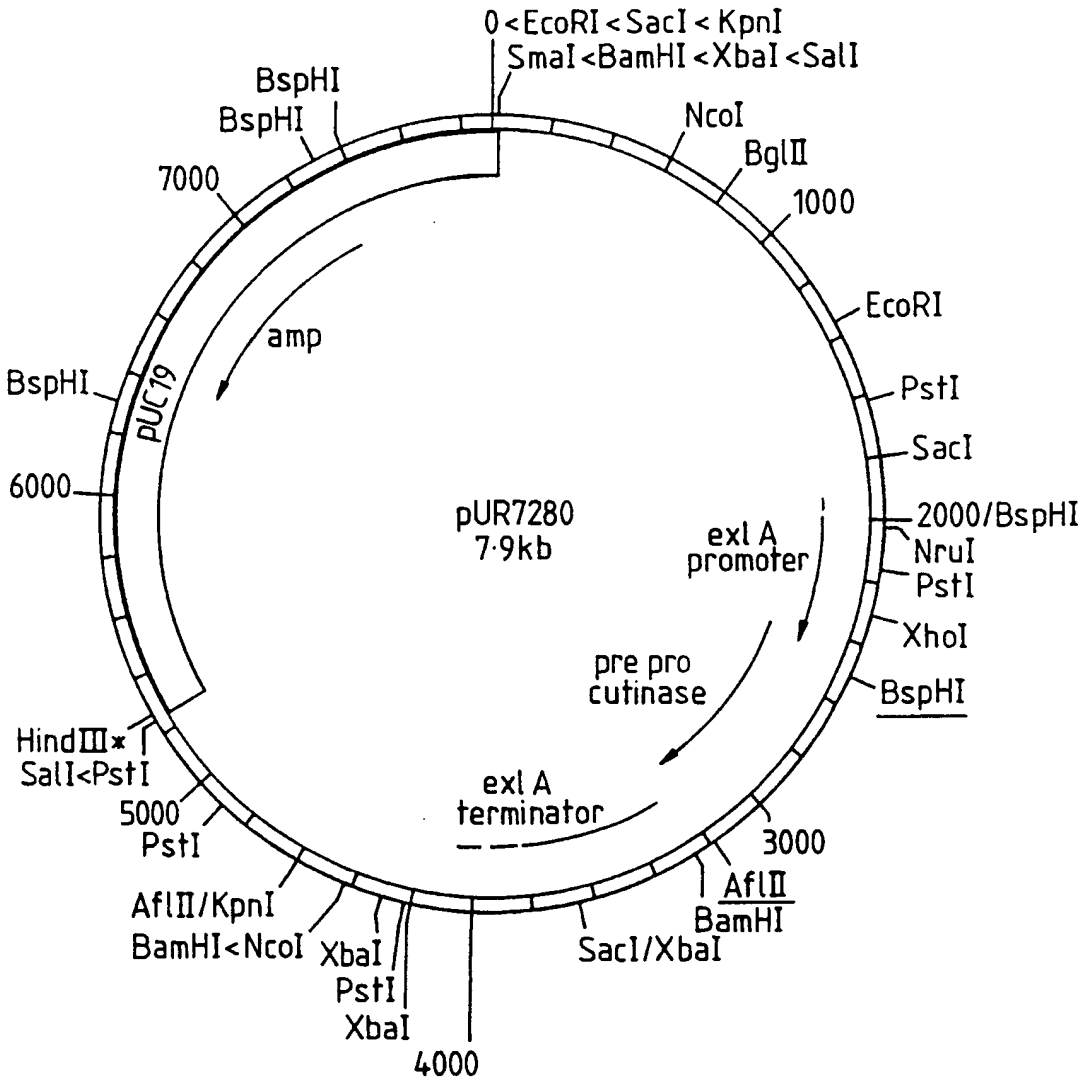

0<EcoRI<SacI<KpnI
SmaI<BamHI<XbaI<SalI
BspHI
BspHI
NcoI
BglII
1000
7000
EcoRI
amp
PstI
BspHI
pUC19
SacI
6000
2000/BspHI
pUR7280
7·9kb
exl A
promoter
NruI
PstI
XhoI
pre pro
cutinase
BspHI
HindIII*
SalI<PstI
exl A
terminator
5000
PstI
3000
AflII
BamHI
AflII/KpnI
BamHI<NcoI
XbaI
PstI
XbaI
SacI/XbaI
4000

Fig.10.

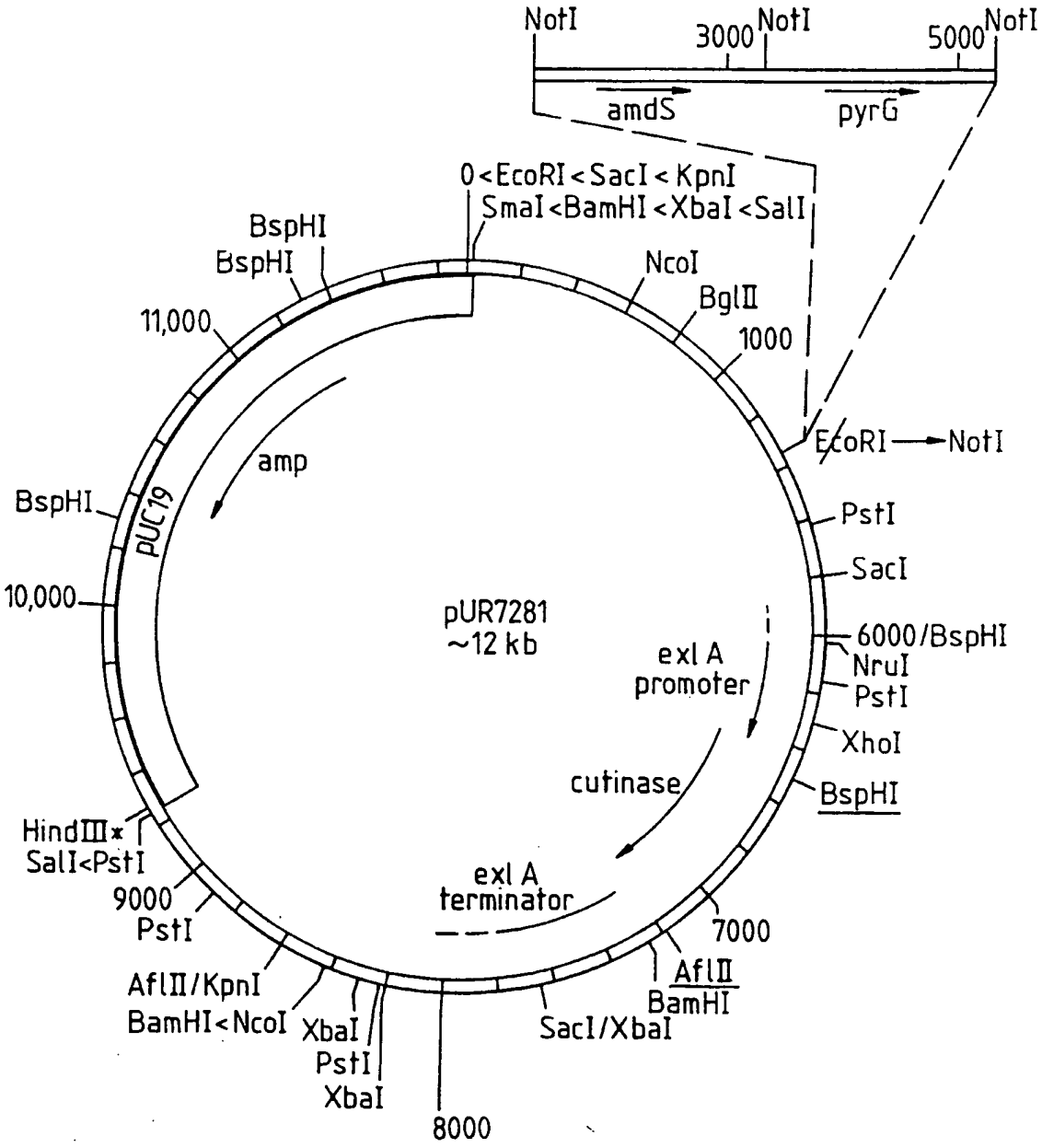

NotI
3000
NotI
5000
NotI
amdS
pyrG
0<EcoRI<SacI<KpnI
SmaI<BamHI<XbaI<SalI
BspHI
BspHI
NcoI
BglII
1000
11,000
EcoRI → NotI
amp
BspHI
pUC19
PstI
SacI
10,000
pUR7281
~12 kb
6000/BspHI
NruI
PstI
exl A
promoter
XhoI
cutinase
BspHI
HindIII*
SalI<PstI
exl A
terminator
9000
PstI
7000
AflII
BamHI
AflII/KpnI
BamHI<NcoI
XbaI
PstI
XbaI
SacI/XbaI
8000

Fig.11.

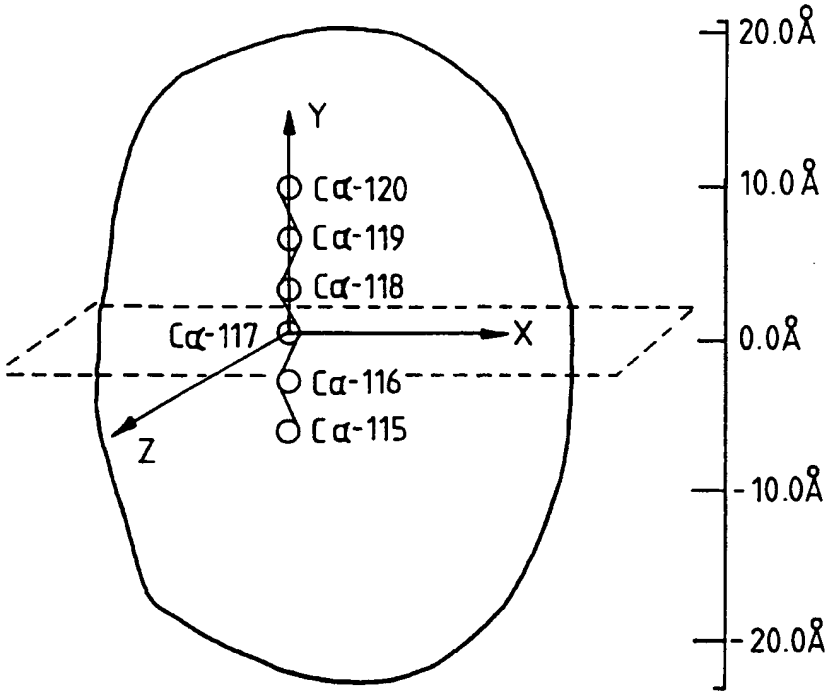

Y
Cα-120
Cα-119
Cα-118
Cα-117
X
Cα-116
Cα-115
Z
20.0Å
10.0Å
0.0Å
-10.0Å
-20.0Å

Fig.12.

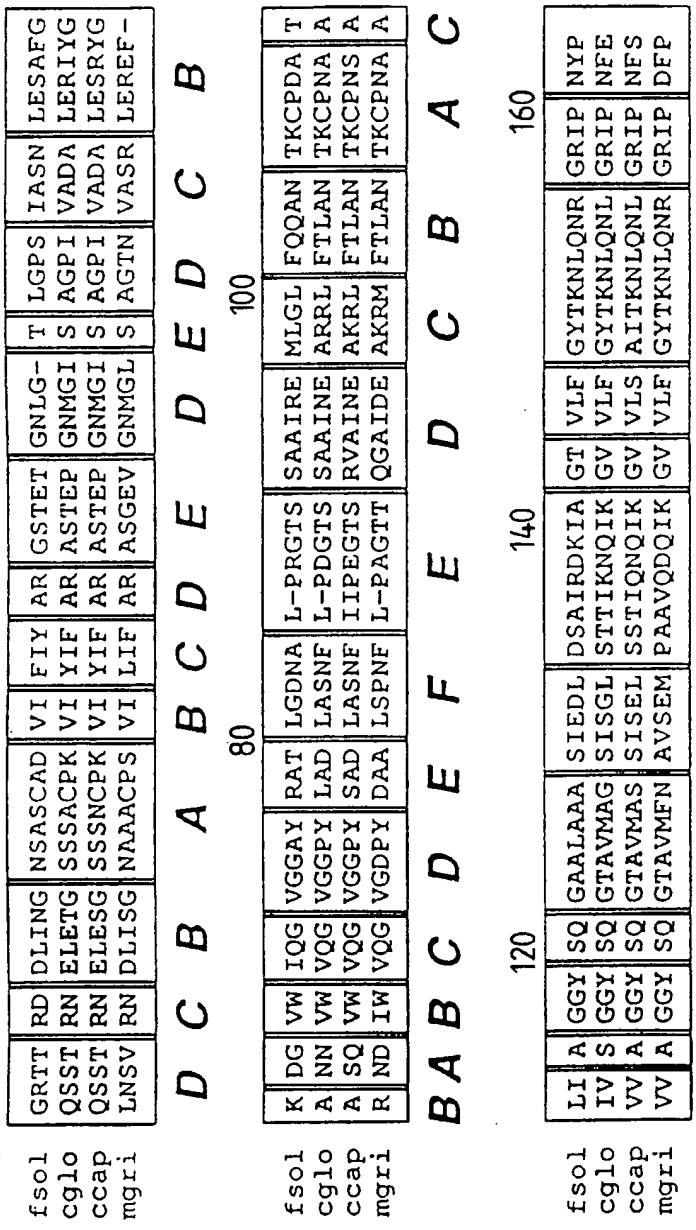

20
40
60
80
100
120
140
160
fsol GRTT RD DLING NSASCAD VI FIY AR GSTET GNLG- T LGPS IASN LESAFG
cglo QSST RN ELETG SSSACPK VI YIF AR ASTEP GNMGI S AGPI VADA LERIYG
ccap QSST RN ELESG SSSNCPK VI YIF AR ASTEP GNMGI S AGPI VADA LESRYG
mgri LNSV RN DLISG NAAACPS VI LIF AR ASGEV GNMGL S AGTN VASR LEREF-
D C B A B C D E D E D C B
fsol K DG VW IQG VGGAY RAT LGDNA L-PRGTS SAAIRE MLGL FQQAN TKCPDA T
cglo A NN VW VQG VGGPY LAD LASNF L-PDGTS SAAINE ARRL FTLAN TKCPNA A
ccap A SQ VW VQG VGGPY SAD LASNF IIPEGTS RVAINE AKRL FTLAN TKCPNS A
mgri R ND IW VQG VGDPY DAA LSPNF L-PAGTT QGAIDE AKRM FTLAN TKCPNA A
B A B C D E F E D C B A C
fsol LI A GGY SQ GAALAAA SIEDL DSAIRDKIA GT VLF GYTKNLQNR GRIP NYP
cglo IV S GGY SQ GTAVMAG SISGL STTIKNQIK GV VLF GYTKNLQNL GRIP NFE
ccap VV A GGY SQ GTAVMAS SISEL SSTIQNQIK GV VLS AITKNLQNL GRIP NFS
mgri VV A GGY SQ GTAVMFN AVSEM PAAVQDQIK GV VLF GYTKNLQNR GRIP DFP
B C D E D C B C D E D C

Fig.12. (Cont.)

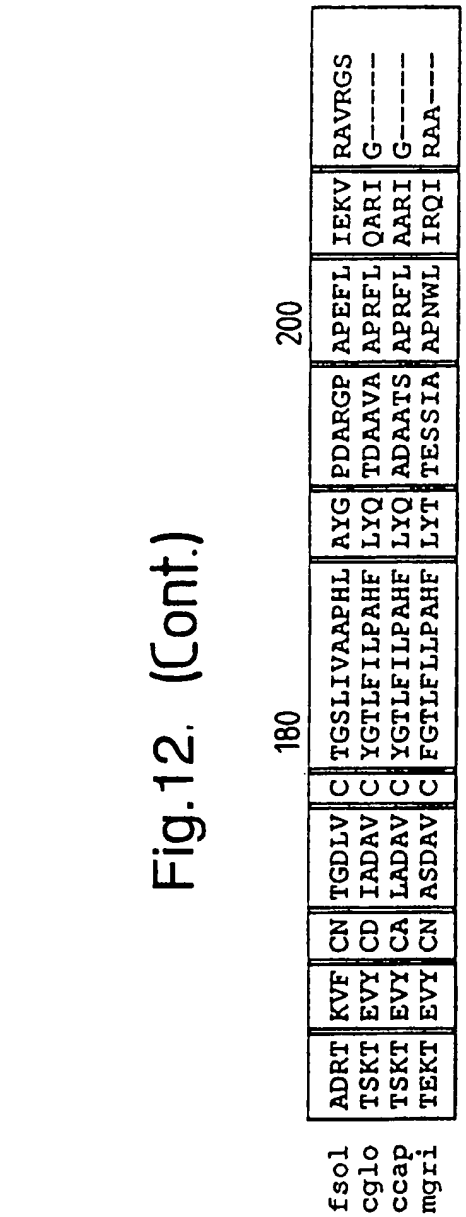

180
200
fsol ADRT KVF CN TGDLV C TGSLIVAAPHL AYG PDARGP APEFL IEKV RAVRGS
cglo TSKT EVY CD IADAV C YGTLFILPAHF LYQ TDAAVA APRFL QARI G-----
ccap TSKT EVY CA LADAV C YGTLFILPAHF LYQ ADAATS APRFL AARI G-----
mgri TEKT EVY CN ASDAV C FGTLFLLPAHF LYT TESSIA APNWL IRQI RAA---
C D E F F E D C B A
range 42 Ångström
top 21 Ångström
bottom -21 Ångström
A: height between -21 and -14 Ångström
B: height between -14 and - 7 Ångström
C: height between - 7 and 0 Ångström
D: height between 0 and 7 Ångström
E: height between 7 and 14 Ångström
F: height between 14 and 21 Ångström

Fig.13.

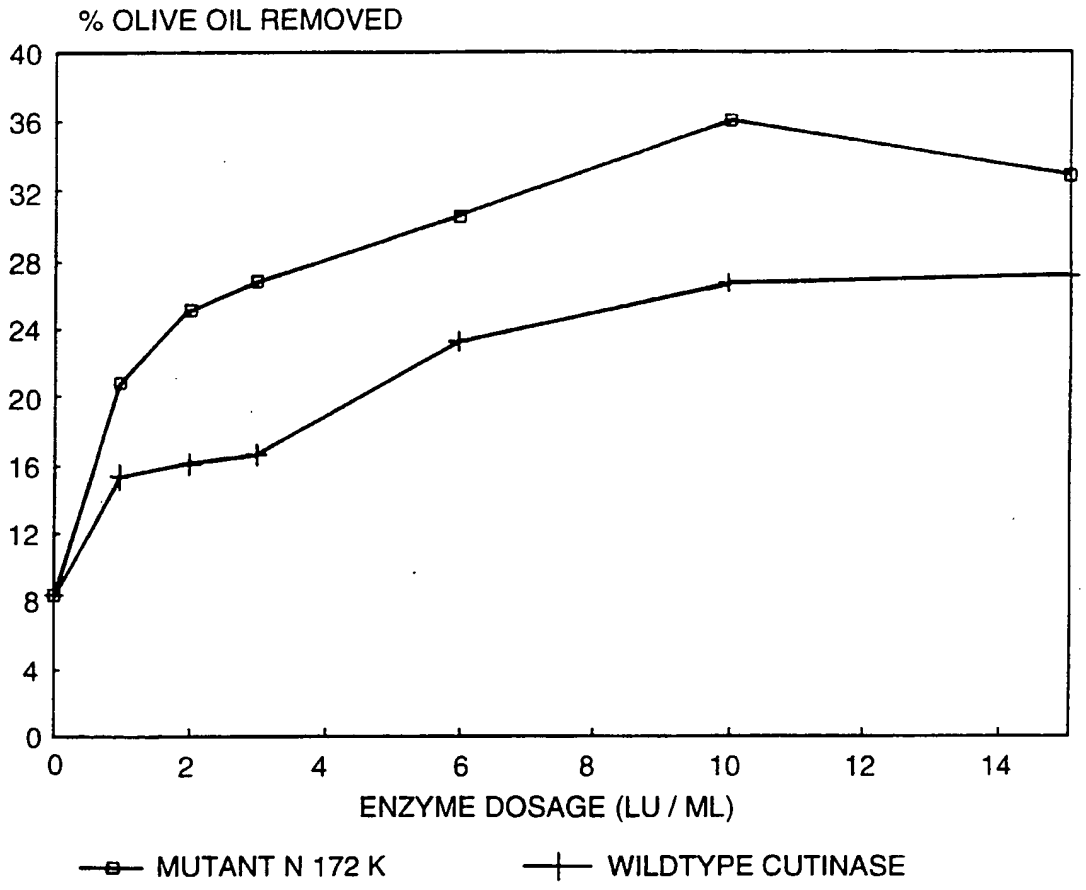

% OLIVE OIL REMOVED
40
36
32
28
24
20
16
12
8
4
0
0
2
4
6
8
10
12
14
ENZYME DOSAGE (LU / ML)
MUTANT N 172 K
WILDTYPE CUTINASE

Fig.14.

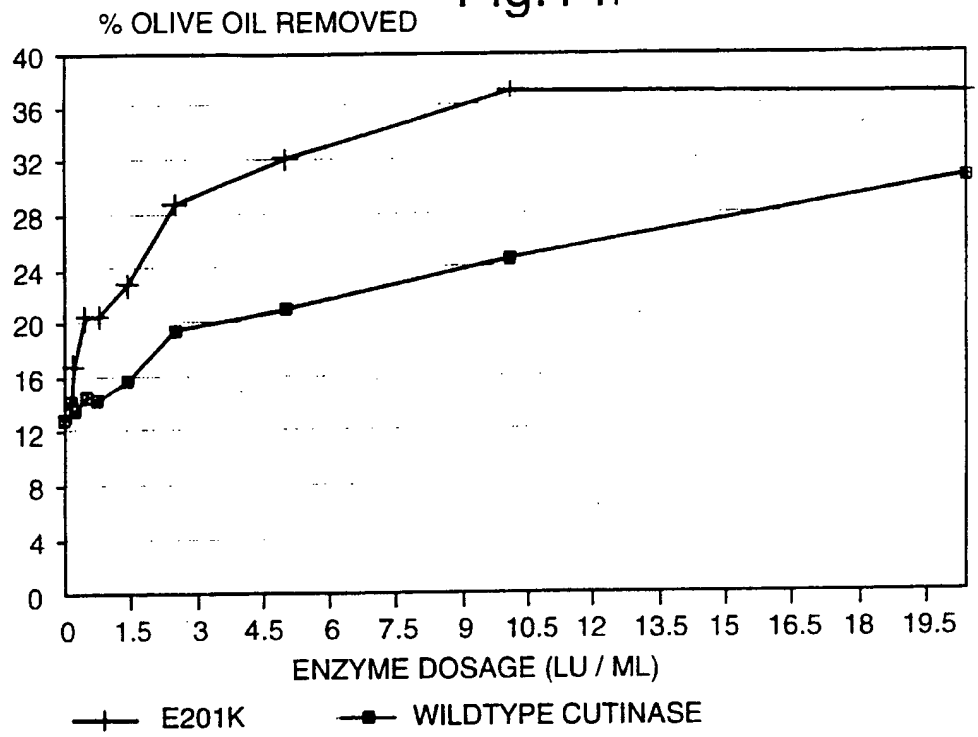
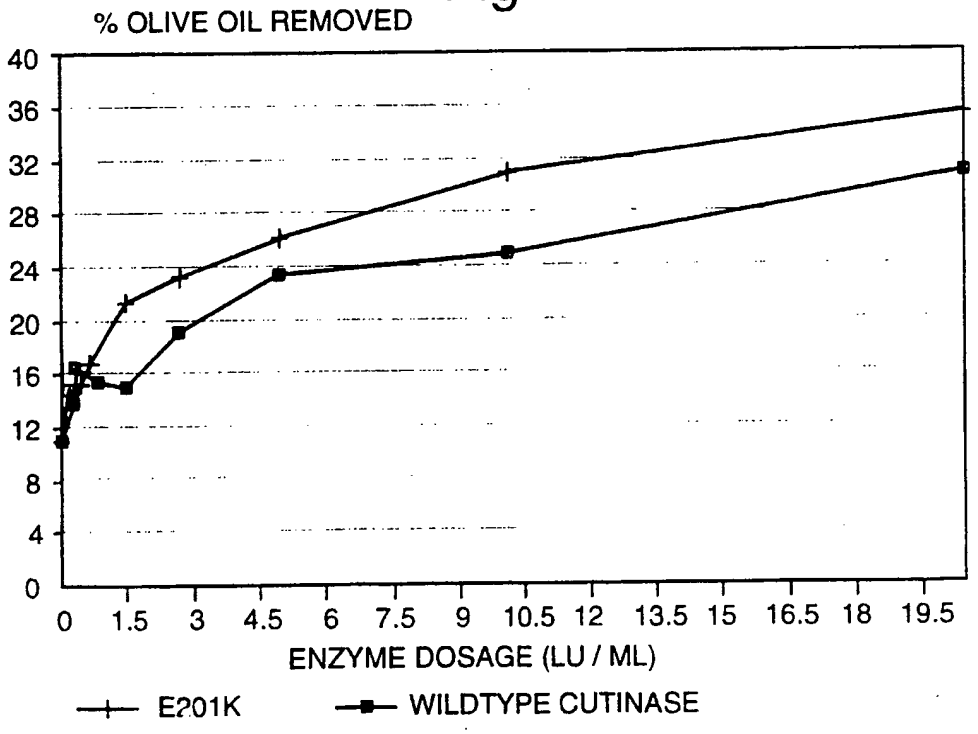

% OLIVE OIL REMOVED
40
36
32
28
24
20
16
12
8
4
0
0 1.5 3 4.5 6 7.5 9 10.5 12 13.5 15 16.5 18 19.5
ENZYME DOSAGE (LU / ML)
E201K
WILDTYPE CUTINASE

Fig.15.


% OLIVE OIL REMOVED
40
36
32
28
24
20
16
12
8
4
0
0 1.5 3 4.5 6 7.5 9 10.5 12 13.5 15 16.5 18 19.5
ENZYME DOSAGE (LU / ML)
E201K
WILDTYPE CUTINASE

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/EP 93/03550

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/55 C12N1/15 C12N1/19 C12N9/18 C11D3/386
C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 05249 (NOVO NORDISK A/S) 2 April 1992 cited in the application see the whole document ---	1-9, 16-28
Y	NATURE vol. 356, no. 6370 , 16 April 1992 pages 615 - 618 MARTINEZ ET AL. 'Fusarium solani cutinase is a lipolytic enzyme with a catalytic serine accessible to solvent' cited in the application ---	1-9, 16-28
A	WO,A,90 09446 (PLANT GENETICS SYSTEMS N.V.) 23 August 1990 cited in the application --- -/--	1-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 April 1994	20. 05. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gac, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROC. NATL ACAD. SCI. USA vol. 81, no. 13 , July 1984 pages 3939 - 3943 SOLIDAY ET AL. 'Cloning and structure determination of cDNA for cutinase, an enzyme involved in fungal penetration of plants' see the whole document	1,8,9, 17,21,25
A	BIOCHEMISTRY vol. 26, no. 24 , 1 December 1987 pages 7883 - 7892 ETTINGER ET AL. 'Structure of cutinase gene, cDNA, and the derived amino acid sequence from phytopathogenic fungi'	1,8,9, 21,25
A	MOL. GEN. GENET. vol. 232, no. 2 , March 1992 pages 174 - 182 SWEIGARD ET AL. 'Cloning and analysis of CUT1, a cutinase gene from Magnaporthe grisea'	1,8,17, 21,25

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/EP 93/03550

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9205249	02-04-92	AU-A-	8617291	15-04-92
		CA-A-	2092615	14-03-92
		EP-A-	0548228	30-06-93
		JP-T-	6501153	10-02-94
WO-A-9009446	23-08-90	NONE		